PHYLOGENETIC RELATIONSHIPS WITHIN THE WESTERN UNITED STATES SPECIES OF *LEPIDIUM* L.

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

in

Phylogenetic Evaluation of Intermountain Lepidium: Interdisciplinary Program

University of Alaska Fairbanks

December 2019

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ABSTRACT

The genus *Lepidium* L. is one of two global genera in the Brassicaceae. The genus has been arranged by species (geographic regions) worldwide, but no formal levels below the genus are recognized. Recent efforts to evaluate phylogenetic relationships have been performed at the global scale for about 20 percent of the species in the genus. The genus is recognized as having subtle and variable morphological characteristics to define species limits. Several nuclear and chloroplast DNA methods have been used to construct phylogenetic relationships within the genus. Incongruences between various phylogenetic trees indicate likely hybridization and/or hybrid origin of multiple species and a genus blurred with a reticulate evolutionary past. Internal Transcribed Spacer (ITS) ribosomal DNA (rDNA) sequences were developed here and combined with other ITS sequences on Genbank for other North American species of *Lepidium*. Two phylogenetic trees were developed, one comparing North American and another dominated by Intermountain West species. Results of a limited Intermountain *Lepidium* phylogenetic tree were compared to a cladistic tree developed from 123 morphological traits for select species of *Lepidium* from the western United States. A comprehensive ITS tree was developed to evaluate species relationships in the genus throughout this region. Ploidy levels of 22 taxa of Intermountain species of *Lepidium* were evaluated to assess whether ploidy levels were associated with any geographic or morphologic patterns within the group. The results show closely related species and varieties with several ploidy levels, but are lacking any relationships to morphological features. Neither ITS nor ploidy levels provided a clear understanding into the current taxonomic treatment of the many faint morphologically different taxa in the group. But Intermountain *Lepidium*, as a geographic group and clade, is distinct from other west coast members in the genus. The species most associated with all the radiant speciation, and the least understood, is L. montana.

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GENERAL INTRODUCTION

The genus Lepidium L., a member of the Brassicaceae, has confounding intrageneric taxonomic rankings resulting from many species having subtle and overlapping morphological characteristics. Within the mustard family, Lepidium is one of two genera having a global distribution, the other being Thlaspi L. (Rollins 1993). Previous authors (Thellung 1906, Bush 1939) speculated that Lepidium was an ancient genus that reached its worldwide distribution in the Late Cretaceous/mid-Tertiary (24-64 mya) with the fragmentation of Laurasia and Gondwana. The fossil pollen record (Muller 1981), the ease of distribution by mucilaginous seeds for intercontinental dispersal by birds (Carlquist 1983; Mummenhoff et al. 1992) and the ease of naturalization to new areas (Al-Shebaz 1986a), do not easily support this interpretation. Recent cpDNA sequence divergence results among the primary Lepidium lineages ranged from 2.1 and 4.2%, with estimated divergence dates ranging from 2.1 to 4.2 mya (Mummenhoff et al., 2001). These levels of divergence place the split of major lineages in the Pliocene (1.8-5.3 mya) during the late Tertiary and not as a vicariance event in the Cretaceous-Tertiary (63-144 mya). Current indications using estimated dates of divergence of cpDNA, coupled with the genus's ability to perform long-distance dispersal by birds (Mummenhoff and Franzke 2007), suggests it to have begun its spread worldwide in the late Pliocene and into the Pleistocene (0.1 to 1.8 mya) (Mummenhoff et al. 2001).

Lepidium and much of the Brassicaceae have their center of distribution in the Iran-Turanian landscape (Thellung 1906, Rollins 1993). This region as a whole is very diverse in altitude, ecological, and geological settings. Most morphological variability contained within Lepidium is found within this region (Mummenhoff et al. 2001). Lepidium habitat globally is typically described as being found in arid and mountainous regions (Thellung 1906). The majority of Lepidium species have maintained this preference for arid habitats, but some species in the genus have adapted and modified their habitat preferences for human-influenced areas worldwide and are recognized as weedy species in many areas (Al-Shebaz 1986b). Both the closely related basal genus Lepia (Desv.) DC and other regional species of Lepidium restricted to Iran-Turanian

territory are reported to be diploids (Lee et al. 2002), whereas most Lepidium of global distribution have a high incidence of polyploidy.

Ploidy levels vary within Lepidium (Al-Shebaz 1986b). One common source of change in ploidy level is hybridization, which has not been reported from field observations, herbarium collections (Hitchcock 1936, 1945, Howell 1934), or experiments (Al-Shebaz 1986b; Lee et al. 2002) for Lepidium. In Lepidium, Warwick and Al-Shebaz (2006) report a base numbers of N = 4, 8, 12, 16, and 32 and 2N = 16, 24, 28, 32, 40, 48, 64, and 80. As discussed by others (Sang et al. 1995, Mummenhoff et al. 2001), genera with incongruencies between topologies of maternally inherited cpDNA in diploids, undetermined hybrids, or ploidy levels in comparison to nuclear ITS sequences as reported in Lepidium (Mummenhoff et al. 2001), suggest a reticulate evolution.

The arid Intermountain West of the United States, with its recent and diverse geologic development and variable topography, has provided excellent opportunities for explosive speciation within Lepidium. Previous efforts of taxonomic classification using morphological features relied heavily on vegetative and fruit characteristics. Many of these previous monographers working within the group using morphological features have noted the need for further clarity at the intrageneric level (Rollins 1993, Holmgren et al. 2005). These faintly distinguished Intermountain taxa have been frequently shuffled within and between various species (Hitchcock 1936, 1950, Rollins 1993, Holmgren et al. 2005), and any distinctions or ability to reasonably develop a phylogenetic interpretation no doubt is blurred by prior multiple hybridizations leading to reticulate evolution (Mummenhoff et al. 2001). The purpose of this study is to undertake a phylogenetic study using nDNA ITS and a survey of ploidy levels and patterns to better understand the evolution, relationships and classification of Intermountain West Lepidium. Information gained from this study will be used to test the hypothesis that Intermountain West and other western United States species of Lepidiums have been involved in repeated hybridization events of crucial progenitor species that have led to the origin of a group of closely related but not highly divergent plants that are adapted to western landscapes

CHAPTER 1: AN OVERVIEW OF PHYLOGENETIC RELATIONSHIPS WITHIN THE WESTERN UNITED STATES SPECIES OF LEPIDIUM L.

ABSTRACT

The genus Lepidium L. is one of two global genera in the Brassicaceae. The genus has been arranged by species (geographic regions) worldwide, but no formal levels below the genus are recognized. Recent efforts to evaluate phylogenetic relationships have been performed at the global scale for about 20 percent of the species in the genus. The genus is recognized as having subtle and variable morphological characteristics to define species limits. Several nuclear and chloroplast DNA methods have been used to construct phylogenetic relationships within the genus. Incongruences between various phylogenetic trees indicate likely hybridization and/or hybrid origin of several species and a genus blurred with a reticulate evolutionary past. Recently, additional ITS DNA sequences developed at UAF were combined with other ITS sequences on Genbank for other North American species of Lepidium and a separate phylogenetic tree was developed. Results of these various phylogenetic trees are compared to a cladistic tree developed from 123 morphological traits for select species of western United States Lepidium. A comparison of the phylogenetic and cladistic trees is used to evaluate methods of selecting the future DNA gene sequence best suited to assess phylogenetic relationships of western United States species of Lepidium.

INTRODUCTION

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The arid Intermountain West of the United States, with its recent and diverse geologic development and variable topography, has provided excellent opportunities for explosive speciation within Lepidium. Previous efforts of taxonomic classification using morphological features relied heavily on vegetative and fruit characteristics. Many of these previous monographers working within the group using morphological features have noted the need for further clarity at the intrageneric level (Rollins 1993, Holmgren et al. 2005). These faintly distinguished Intermountain taxa have been frequently shuffled within and between various species (Hitchcock 1936, 1950, Rollins 1993, Holmgren et al. 2005), and any distinctions or ability to reasonable develop a phylogenetic interpretation no doubt is blurred by prior multiple hybridizations leading to reticulate evolution (Mummenhoff et al. 2001). Therefore, this review is undertaken to summarize the molecular genetic literature that addresses Lepidium so as to take advantage of global efforts in support of a better understanding of the evolution, relationships and classification of Intermountain West Lepidiums. Using information gained from this review along with exploratory results, a directional study design will be developed to establish the methods to test the hypothesis that Intermountain West and other western United States species of Lepidiums have been involved in repeated hybridization events of crucial progenitor species that have led to the origin of a group of closely related but not highly divergent plants that are adapted to western landscapes.

MOLECULAR METHODS

The use of DNA phylogenetic methods has been on the increase since the late 1980s (Avise 1993). DNA analyses techniques have allowed researchers to investigate genetic controls on morphology, physiology, disease, and phylogenetics at the most basic DNA level. These recently developed technologies have revolutionized the systematics and classification of interpretations surrounding organismal taxonomy (Olmstead 2002). Of the many DNA techniques used in biological research, those most frequently used in Lepidium are reviewed here.

Internal Transcribed Spacer (ITS)

ITS DNA analysis uses the nuclear ribosomal RNA (rDNA) region of the genome. These genomic analyses have also provided sources of nuclear DNA characters for inferring intra- and intergeneric evolutionary relationships in plants (Hillis and Dixon 1991; Hamby and Zimmer 1992). ITS sequencing relies on rDNA strands arranged in repeating units that are found in high and variable copy number. Unlike the highly conserved gene regions of the RNA, ITS regions are highly variable in genetic code and have evolved at different rates of divergence throughout the ITS region. These variations and divergences in code are the basis of the genetic sequence information used in phylogenetic studies, but here are some recognized limitations to ITS. Species derived from hybridization and polyploidization have their parental lines homogenized by concerted evolution, or intergenic sequence exchange in the ITS region, and eventually express only one line of parental origin in the hybrid (Avise 1993). Species of hybrid origins and reticulate patterns of evolution can be problematic for ITS analysis because of this limitation. However, Sang et al. (1995) constructed phylogenetic relationships in Peonies species derived through hybridization and polyploidy. They and others (Hillis and Dixon 1991) have cautioned that concerted evolution within gene families in this genomic region may obscure patterns of nucleotide additivity in the ITS sequences of hybrids when applying these data to phylogenetic studies.

Chloroplast DNA (cpDNA)

cpDNA is transmitted maternally in most plants (Gilham 1978, Hachtel 1980), biparentally in some (Metzlaff et al. 1981), and paternally in several gymnosperms (Szmidt et al. 1987, Wagner et al. 1987). These cpDNA sequences are reported to have, in the ancestral past, occasionally moved to the nucleus and exchange with sequences within mtDNA and nDNA (Nugent and Palmer 1991, Stern and Palmer 1984). These and other features have led to them being highly employed in systematic studies using cpDNA. The advantages of using cpDNA are: 1) lot of published information on cpDNA use, which is useful for experimental and comparative studies; 2) cpDNA genes are well suited for cladistic type studies; and 3) it uses a conservative rate of nucleotide substitution rate (Ritland and Clegg 1987; Clegg and Zurawski 1992). Thus, cpDNA has been used extensively to document reticulate evolution resulting from interspecific hybridization and introgression between closely related species. This same strength to detect parental additivity is also misleading and may not be congruent with nucleic phylogenetic evolution since that nucleic DNA represents the evolution of the species in general and not just the chloroplast.

In Lepidium, previous studies using cpDNA focused on restriction site analysis (Mummenhoff and Koch 1994; Koch et al. 1998), sequencing of coding regions such as rbcL, atpB, matK, ndhF, and rpl16 (Chase et al. 1993; Olmstead and Palmer 1994; Olmstead and Sweere 1994; Steele and Vilgalys 1994; Hoot et al. 1997; Schnabel and Wendel 1998) or non-coding regions like trnL spacers and introns (Small et al. 1998).

Amplified Fragment Length Polymorphism (AFLP)

AFLP is reported to have the reliability of RFLP in conjunction with the combined power of PCR techniques (Vos et al. 1995) and is very sensitive to molecular genetic variation that exists between closely related species or varieties. The AFLP fragments of DNA are amplified from restriction digested genomic DNA (Matthes et al. 1998; Karp et al. 1997). AFLPs have been highly used in genomic mapping and the construction of high-density genetic maps (Vos et al. 1995). With the capability to detect high levels of polymorphism, AFLPs are frequently used for

population-level studies to detect basic biological diversity and genetic variation. AFLPs have been used in phylogenetic analyses to demonstrate genetic variation of ecotypes (Russell et al. 1999), introgression, and hybridization (Riesberg et al. 1999) at the species level. The greatest advantages of AFLPs are their ability to detect high levels of polymorphism and their repeatability. These abilities allow detection of differences between individuals (Law et al. 1998). Possible disadvantages include the selection of primers and the fact that restrictive enzymes can affect the number of AFLP polymorphisms, such that the variation detected can be influenced by the choice of primers selected. Homology is a concern with AFLP analysis since co-migrating bands are assumed to be homologous, but there is no precedence for that assumption (Mace et al. 1999).

Chalcone Synthase (Chs)

Chalcone synthase (Chs) is a nuclear gene that plays a central role in the secondary metabolism of flavonoid biosynthesis. It has been shown to be extremely useful in phylogenetic studies of the Cruciferae (Koch et al. 2001). Nuclear genes are particularly helpful in certain phylogenetic studies because they can provide evidence for the affinities of homologous genomes in allopolyploid taxa with their diploid progenitors (Doyle and Doyle 1999). Chs is recognized as being an important source of phylogenetically useful characters in polyploids because they often have variable intron sequences that can be used to dissect the relationships among closely related taxa (Small et al. 1998; Shimizu et al. 2005).

Pistillata (PI)

Pistillata (PI) acts to specify the development of the petals and stamens normally found in the second and third flowers whorls (Bowman et al. 1999, Lee et al. 2002, Goto and Meyerowitz 1994). It is a single-copy gene. Previous studies on the phylogenetic utility of PI first intron sequences in Brassicaceae showed that it provided more phylogenetically informative characters than either ITS or cpDNA, and it consistently supported species relationships when compared to other markers (Bailey and Doyle 1999). An advantage of this sequence is its ability to detect hybrid parentage in allopolyploid species (Lee et al. 2002).

PHYLOGENETIC OVERVIEW OF BRASSICACEAE AND THE INTERMOUNTAIN WEST LEPIDIUM

To date, most of the published phylogenetic efforts, both in the Brassicaceae and Lepidium, have focused on a worldwide perspective (Mummenhoff et al. 1992, 2001, Koch et al. 2001, Lee et al. 2002). The majority of current research reporting deals with either tribal classification within the family or global grex (geographic) groups previously described by Thellung (1906) within the genus Lepidium. There are several other lines of research within Lepidium that focus on other topics, such as modified floral patterns, floral gene control, and phylogenetic global associations of floral design (Bowman et al. 1999). Other lines of study involving phylogenetic approaches have dealt with speciation patterns influenced by allopolyploidy and detected by various DNA analysis methods.

Each of these phylogenetic study efforts generally includes a few Intermountain West species of Lepidium. But to date there is no published comprehensive phylogenetic overview of North American Lepidiums or any "natural sections" or geographic group of species, such as those in the Intermountain region. Those species of Lepidium included in global studies from across the western United States (U.S.) and North America are generally distant or unrelated taxa. There are efforts underway to develop phylogenetic relationships for all species of Lepidium that will support the taxonomic treatment of the genus at only the species level in the flora of North America (John Gaskin 2007, pers. com.). Gaskin's unpublished efforts use ITS sequencing. More recently there has been an effort to assess the genetic distinction of the proposed endangered species L. papilliferum (L.F. Hender.) A. Nelson and J.F. Macbr. from Idaho (Larson et al. 2007, draft). Larson's efforts included ITS, cpDNA, and AFLP DNA sequencing methods. In those efforts, Larson showed that L. montanum and L. papilliferum are distinct from each other, with L. papilliferum having two geographically genetic regions that are not sympatric. However, Larson noted in passing that genetically the most similar alignment to L. papilliferum was L. fremontii, and further efforts were needed to resolve relationships within the L. montanum group.

Perspectives of Current Intermountain West Lepidium Phylogenetics

A phylogenetic relationship of the western U.S. and the Intermountain West species of Lepidium is limited. To initiate a phylogenetic understanding of Intermountain West species, phylogenetic trees from the literature were condensed to those species from North American and possible related species from Eurasia (Fig. 1.1, 1.2, and 1.3). With minor modifications, the reduced trees presented below (Figs. 1.1-1.3) were replicated to most closely duplicate the published and larger trees from which they were developed.

In three phylogenetic trees using different genes, there are incongruences between several clades. The ITS phylogenetic relationships of seven western U.S. species sorted into two separate clades (Fig. 1.1). Clade C, labeled California, contains four species with distribution patterns mainly from the west coast of the U.S. and into Nevada. Clade B was a mix of California and other western U.S. species that aligned with more widespread and weedy species found throughout North America. In clade B, L. flavum Torr. splits at an equal node that sorts sister species L. fremontii S. Watson and L. montanum Nutt. The cpDNA sorted L. fremontii, L. montanum, and L. flavum together in the same final branch in clade A (Fig. 1.2). Similar to ITS, cpDNA placed these three western U.S. species closer to more widespread species from across North American. Eurasian species also linked phylogenetically to North American Lepidiums (Mummenhoff et al. 2001) in clade A, including both western U.S. and more widespread North American species. The California-restricted Lepidiums aligned in their own sub-clade. In ITS, the Eurasian species were a separate clade (Fig. 1.1).

Pistillata (PI) shows some similar alignments but divides the western species into two clades. In the PI phylogenetic alignment, western species of L. fremontii, L. montanum, and L. flavum were located in both major clades A and C (Fig. 1.3). Again, L. montanum and L. flavum aligned with more widespread Lepidiums of clade A from North America. Likewise, L. fremontii and L. montanum aligned with other widespread Lepidiums in the other major clades C and D. Interestingly, L. montanum occurred twice, once in clade A with L. flavum and again in clade C with L. fremontii. The Eurasian species L. lyratum L. aligned with the western U.S. and widespread Lepidiums of clades A and B.

UAF PRELIMINARY DATA

Phylogenetic Data

Exploratory ITS sequencing from several western Lepidiums adds another set of phylogenetic information to the western U.S. complex. By combining ITS sequences posted on Genbank, it was possible to develop two additional phylogenetic trees with some of the same North American and Eurasian species of Lepidium shown in Figures 1.1–1.3. Construction of a comprehensive ITS tree was limited because ITS sequences posted on Genbank were of two types, those from either ITS1 or ITS2 sequences. This limited the comparison of all species in one phylogenetic analysis based on the ITS2 sequence. L. virginicum L. was selected as the outgroup for analysis based on its global distribution pattern with a distant relationship to western U.S. species and differing morphological character states.

Several phylogenetic patterns are similar to those presented by Mummenhoff et al. (2001) and Lee et al. (2002) for both western U.S. and widespread North American Lepidiums in both ITS1 and ITS2 trees (Figs. 1.4 and 1.5). Likewise, the Eurasian species aligned into a separate clade. In ITS2, the Eurasian clade A is near the western U.S. Lepidiums. Since L. flavum was only sequenced with ITS2, it only appears in that phylogenetic tree (Fig. 1.4). But in the ITS2 analysis, L. flavum branches near and after the clade division leading to relationships between L. fremontii, L. papilliferum, L. montanum, and L. crenatum (Greene) Rydb. Western U.S. species were grouped with a high bootstrapped value of 99%. In the final division of the western U.S. clade C, there is a split between L. montanum and L. crenatum from L. papilliferum and L. fremontii. Though the bootstrap values were less than 75%, L. papilliferum and L. fremontii have a value of 66%, and L. montanum and L. crenatum with 69%.

Discussion of Phylogenetic Relationships

Differences between nuclear and cpDNA sequence capabilities become evident when comparing this series of phylogenetic trees in Lepidium. Both the ITS and cpDNA phylogenies (Figs. 1.1 and 1.2) place the western species of L. fremontii, L. montanum, and L. flavum in similar relationships within their respective clades. ITS (Fig. 1.1) did split L. flavum at the same node as

the branch leading to L. fremontii and L. montanum, which indicates a divergence between these three species. PI (Fig. 1.3) sorted L. montanum into two separate clades, once as a sister species with L. fremontii and then again with L. flavum. Lee et al. (2002) reports that L. montanum is a tetraploid having a partial parental lineage with L. fremontii. The PI results reported by Lee et al. (2002) indicate a paraphyletic origin of L. montanum. The paraphyletic L. montanum with different sister species in each separate clade suggests a probable parental hybridization origin derived from L. flavum and L. fremontii.

Likewise, the UAF ITS2 results (Fig. 1.4) are monophyletic for L. fremontii and L. papilliferum. The ITS2 sorting of L. papilliferum and L. fremontii together in a monophyletic clade that includes L. montanum is incongruent with results for L. fremontii in cpDNA and PI. Sang et al. (1995) suggests that such incongruencies imply a reticulate evolutionary past involving hybridization. With L. papilliferum being reported as an allopolyploid (Stillman et al. 2005), one possible hybridization event may be between L. montanum and L. fremontii. Another possible hybrid origin of L. papilliferum may involve the same set of parents (L. fremontii × L. flavum) but from a different hybridization event or time (Soltis and Soltis 1993). It's also possible that the L. papillerifum hybridization event involved a possible backcrossing event between one set of possible parents, that of L. flavum and L. fremontii.

Morphological Data

To better understand the currently held morphologically based species concepts associated with the Intermountain West Lepidiums, 123 morphological characters were analyzed using a discrete character parsimony algorithm (PARS) (Felstenstein, Univ. Washington; http://evolution.genetics.washington.edu/phylip/doc/pars.html). The PARS algorithm is intended to produce the shortest parsimony branches of most likely evolutionary relationships (Hochbaum and Pathria 1997). Morphological characteristics were tallied from 2007 voucher specimens (collections of R. Lichvar) and combined with written technical descriptions provided by Rollins (1993), Holmgren et al. (2005), and Hitchcock (1936). The cladogram for Intermountain West Lepidiums is divided into two clades (Fig. 1.6). Clade A includes two cushion plant species (L. davisii and L. barnebyanum), a large mostly entire leaf species (L. intergrifolium), and a

geographically disjunct but similar appearing species to L. montanum. This clade has a wide variety of morphological types and appears to be paraphyletic based on those 123 morphological characteristics.

All species in clade B have been treated by various authors as varieties under L. montanum except for L. fremontii and L. huberi. L. huberi has been recently described (Welsh and Goodrich 1995) and therefore has not been subject to prior revisions by various authors who may well have treated it also at the variety level. The lineage demarked by L. huberi through L. alyssoides branches all share a large growth stature with highly branched stems. It is difficult to interpret whether or not the traits used to distinguish species in this clade are derived or convergent since a larger and wider-ranging group of species needs to be further studied for comparative purposes.

Using morphological traits, the Intermountain species sort into reasonable morphological-based clades of sister species. There has been no effort to further separate Lepidium at a recognized taxonomic ranking below the genus level, other than to species or varieties. In Lepidium, the lack of subgroups may indicate that the monographers felt that the faint and widely variable morphological nature of most of the traits lacked strong and clear distinction. In treating western U.S. species, both Rollins (1993) and Holmgren et al. (2005) remarked about the lack of understanding for the relationships that exist between L. barnebyanum and L. davisii, L. papilliferum and L. montanum, and the treatment of L. huberi through the L. alyssoides branch. These gaps in our knowledge hinder our ability to confidently treat various taxa at the species or varietal level. Statements about relationships between select species made by Rollins (1993) and Holmgren et al. (2005) are reinforced when noting the incongruency between the cladogram and the nuclear ITS results that clearly indicate that some of these species noted by these authors were more closely related to other species as identified using phylogenetic approaches.

An interesting alignment in PARS resulted as the placement of L. fremontii into clade B. This species is on the eastern edge of its range in the Intermountain region and is more western in its distribution into California. In the cladistic analysis, this species is the only member of the group that has a style that is sessile within the silique notch. All other species in the cladogram, except

for the outgroup species L. virginicum, have styles that exceed the notch. This feature hasn't been used to divide the genus into subgroups, but it has been used when making general references to various species that are in the "exceeds the notch" fruiting group. In this analysis, fruiting traits used in combination with its other features were not significant enough to force a separation of L. fremontii into its own clade.

PHYLOGENETIC AND CLADISTIC ANALYSIS DISCUSSION

Several differences are noted within and between phylogenetic and cladistic analyses. As discussed in phylogenetic relationship section, ITS and cpDNA analyses were not able to detect or indicate the possible allopolyploid origin for L. montanum from L. flavum and L. fremontii as was detected by PI. In the cladistic analysis, L. fremontii aligned in another clade and did not align with L. montanum. Another incongruency occurred with L. crenatum. In the UAF ITS2 phylogenetic analysis, L. crenatum closely aligned with the L. montanum-L. papilliferum-L. fremontii group. In the cladistic analysis, L. crenatum sorted into an entirely different clade along with the larger bushier members of the L. alyssoides group.

Incongruencies between phylogenetic and cladistic analyses are significant. These differences are problematic for several reasons: 1) attempting to classify western species of Lepidium by morphological features alone is not possible due to the potential for convergent evolution of multiple traits, 2) the phylogeny of this group cannot be developed solely from morphological or cladistic approaches because of the inability to detect allopolyploid origins involved with reticulate evolution, and 3) without an understanding of hybrid origins of many species and possible varieties, the reliance on morphological traits for assigning taxonomic ranking is not possible with any level of confidence in Lepidium.

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CHAPTER 1 FIGURES

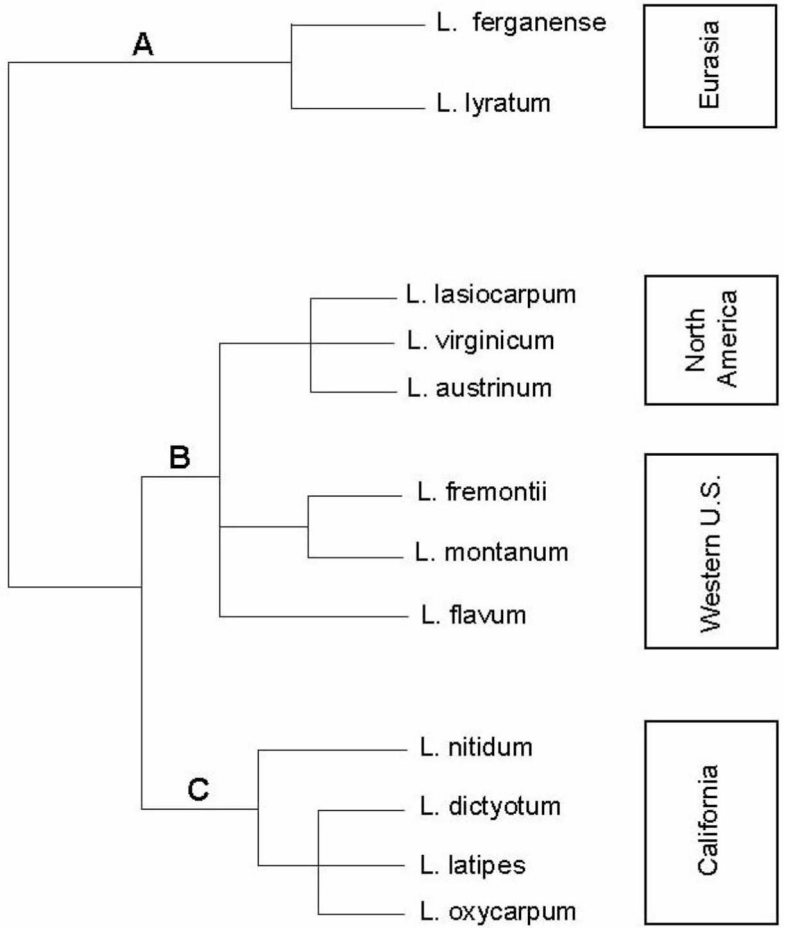

Figure 1.1. ITS analysis of western United States Lepidiums (modified from Mummenhoff et al. 2001).

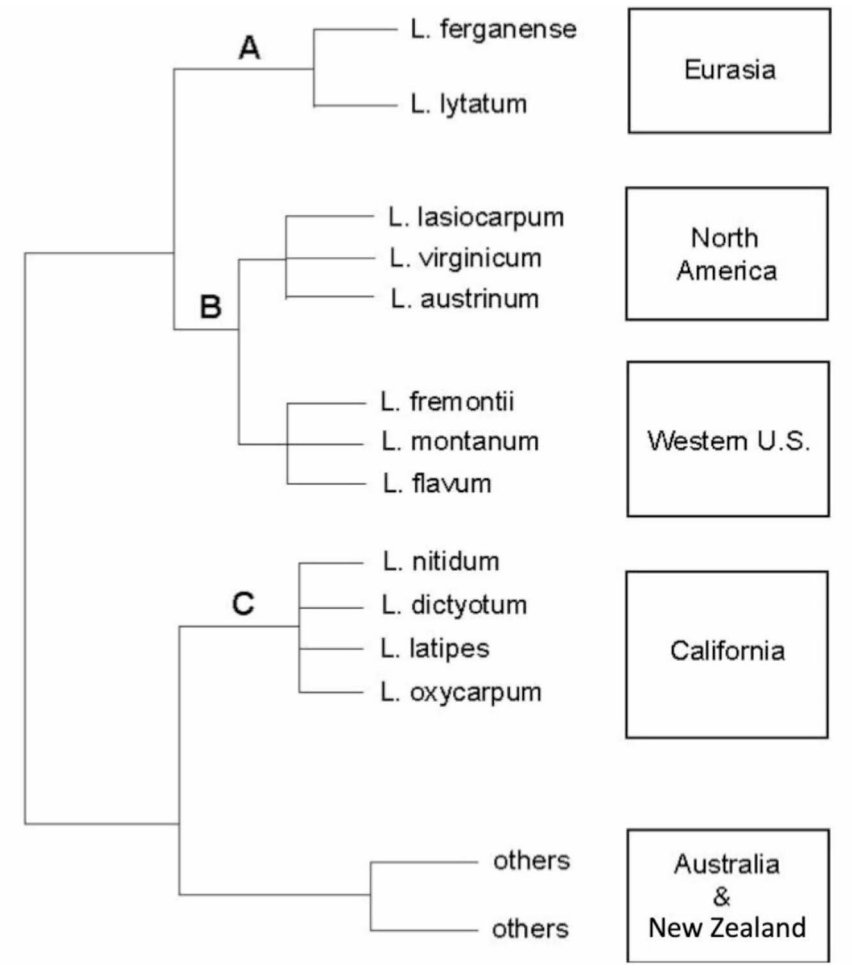

Figure 1.2. cpDNA of western United States and several global species of Lepidium (modified from Mummenhoff et al. 2001).

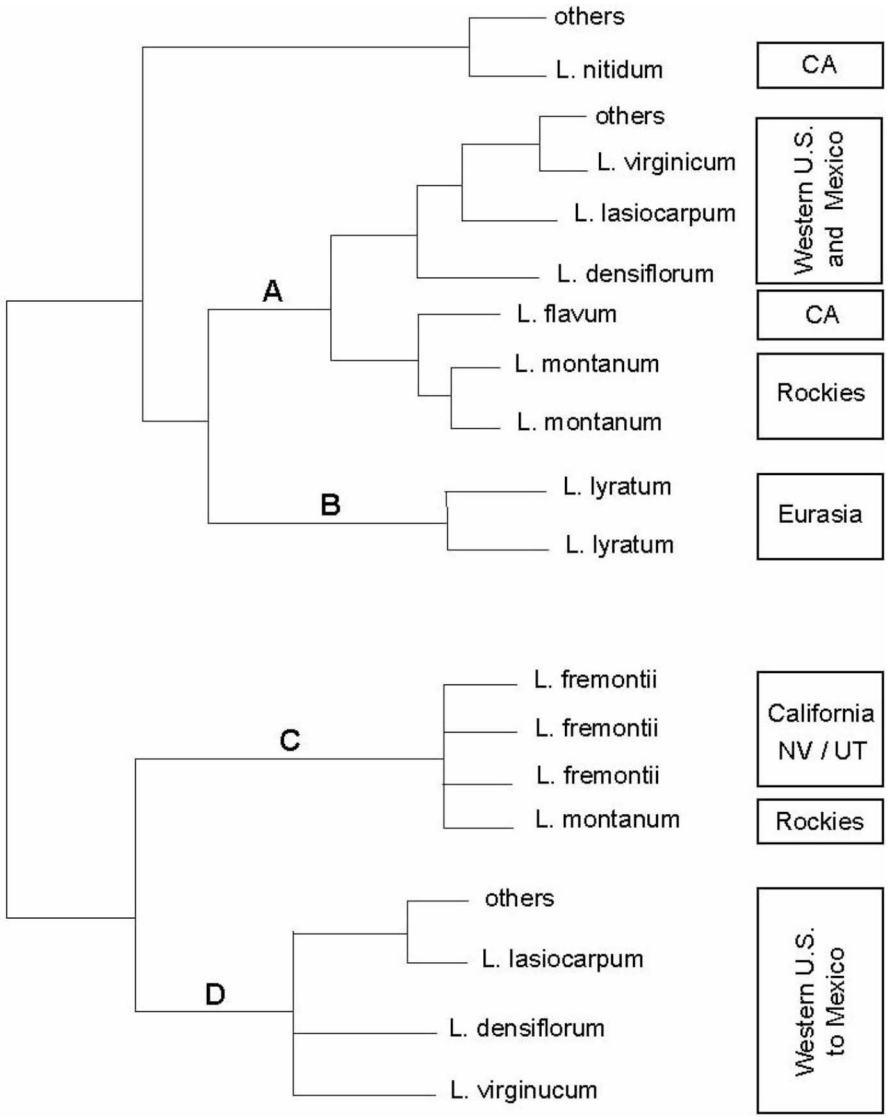

Figure 1.3. Pistillata analysis of selected western Lepidiums (modified from Lee et al. 2002).

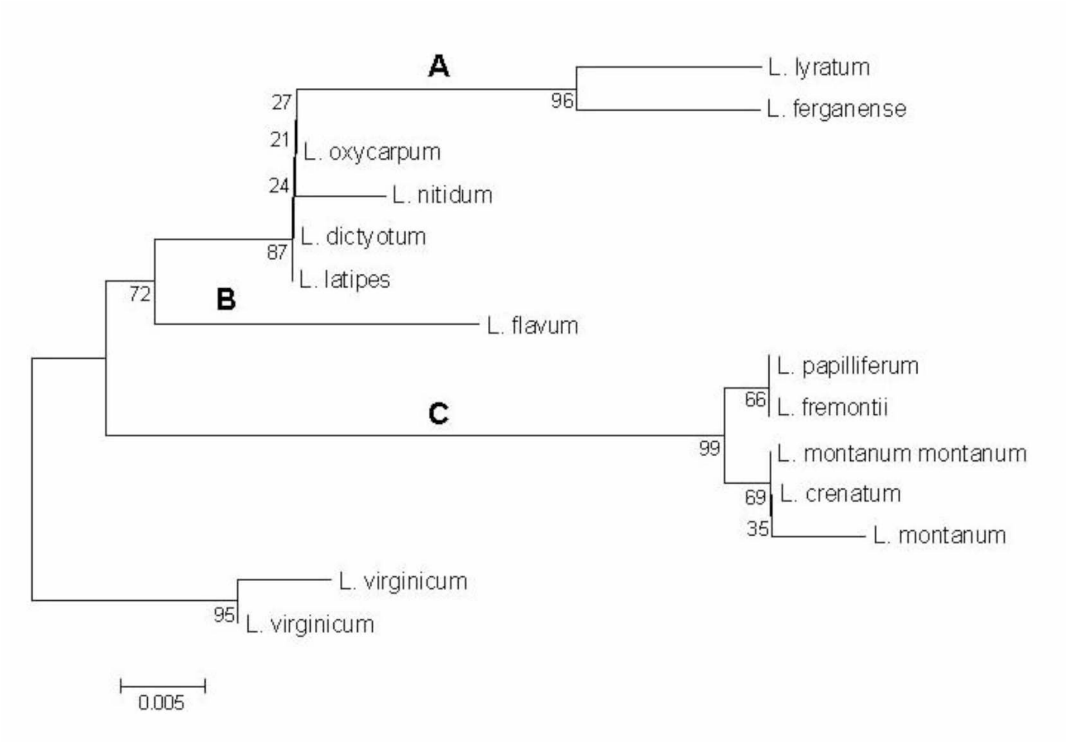

Figure 1.4. ITS2 neighbor joining tree. Species are a combination of ITS sequences downloaded from Genbank and recent UAF exploratory ITS2 sequences.

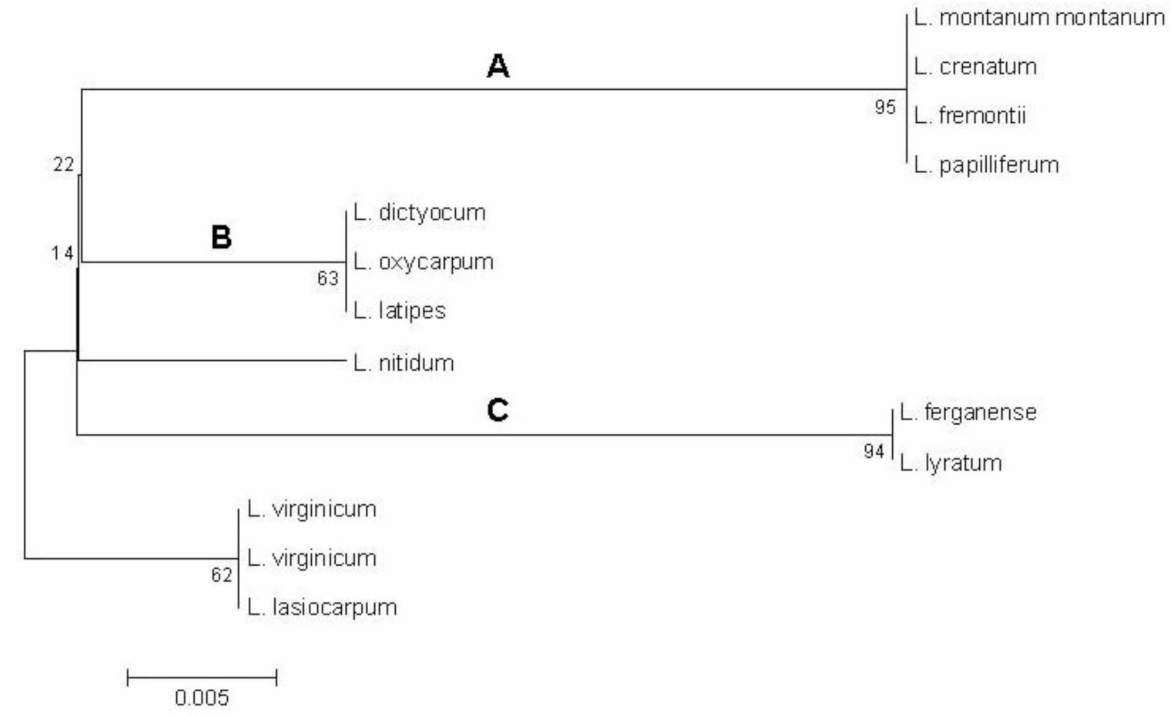

Figure 1.5. ITS1 neighbor joining tree. Species are a combination of ITS sequences downloaded from Genbank and recent UAF exploratory ITS1 and ITS2 sequences.

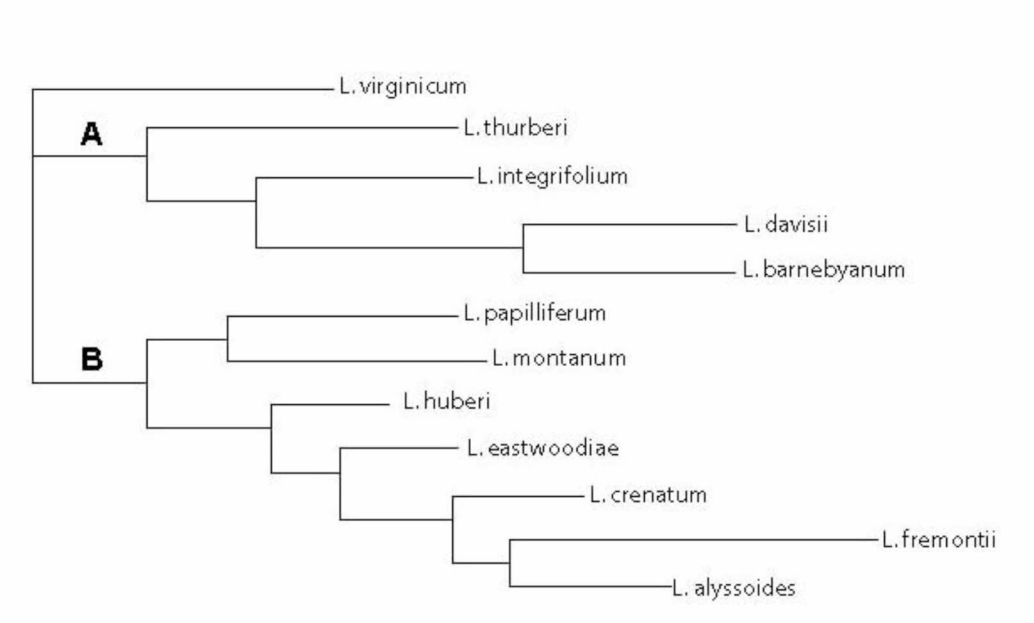

Figure 1.6. PARS cladistic tree based on the use of 123 morphological character states.

CHAPTER 2: GENOMIC SIZE AND PLOIDY LEVEL PATTERNS OF INTERMOUNTAIN WEST LEPIDIUM USING FLOW CYTOMETRY

ABSTRACT

The taxonomic status of members of the genus Lepidium in the Intermountain West has been in flux for years. Species concepts and classification of these endemic species from the western United States centers on the highly variable L. montanum complex. Until recently, classification treatments using morphological features in this group have been adequate, but as more new species are discovered and more locations reported, the limited number of morphological features available for classification and defining species concepts has led to more uncertainty about taxonomic rankings. As part of a molecular and morphological-based treatment of the group, flow cytometry was used to evaluate 58 collections of Lepidium from the Intermountain West. The survey of ploidy level for 14 species and 11 varieties of Lepidium was initiated to assess whether ploidy levels were affecting the interpretation of taxonomic rankings. Of those Lepidium surveyed, 90% were found to be tetraploids, along with several taxa that are diploid and hexaploid. Tetraploid occurrences crossed all major species and varieties. No geographic distribution or habitat patterns were found to be associated with ploidy level.

INTRODUCTION

The Brassicaceae is distributed worldwide and consists of 49 tribes, 321 genera, and more than 3660 species (Al-Shehbaz 2012). The use of chromosome numbers for distinguishing differences between genera in the Brassicaceae has been limited (Rollins 1993). Even though the chromosome numbers are not taxonomically definitive in Brassicaeae, in some cases the numbers are consistent and not random among different species groups. Rollins (1955, 1979, 1993) showed that certain species groups within select genera share a similar base number and in some cases are associated with groups with specific morphological characteristics. As such, chromosome numbers have been supportive of taxonomic treatments in Brassicaceae between some species (Rollins 1963, Mulligan 1976) and have been found not to be useful in others

(Mulligan 1966). As a whole, chromosome counts across the family express frequent occurrence of hybridization, apomixes, and variations in ploidy levels and therefore may or may not be supportive for taxonomic purposes.

Base chromosome numbers in the family vary from x = 4 to 17, with more than one-third of the taxa having karyotypes of x = 8 (Warwick and Al-Shehbaz 2006). Chromosome counts reported by Warwick and Al-Shehbaz (2006) represent 232 of the 321 genera and 1558 of the 3660 species in the Brassicaceae. Of those reported, approximately 37% of the species are assumed to be polyploids. These estimates may be even higher if diploid species are shown to be paleopolyploids that formed from diploidization (Lysak et al. 2005). Complicating the utility of chromosome numbers even further are the frequent auto- and alloploid events followed by chromosome reshuffling, fusions, and fissions, which have caused intra-generic numeric variation and/or descending or ascending dysploidy (Lysak et al. 2005), making the base number impractical for taxonomic interpretation in some genera. In the Brassicaceae, x = 4 is the lowest base number and is found in two unrelated genera—Stenopetalum R. Brown from Australia and Physaria (Nutt.) A. Gray. The highest base number is x = 128, found in Cardamine (Easterly 1963; Al-Shehbaz 1988). In Lepidium L., the base number is reported as x = 8 based on 231 counts from 60 species (Warwick and Al-Shehbaz 2006). In the reported counts for Lepidium, 34% were diploid, 14% both diploid and polyploid, and 52% entirely polyploid. Ploidy numbers in the genus are reported as 2n = 16, 24, 28, 32, 40, 48, 64, and 80.

In addition to counts of chromosomes numbers for supporting the evaluation of ploidy levels, the amount of DNA per nucleus can now be determined using techniques such as flow cytometry (FC) (Bennett and Leitch 2005, Doležel and Bartoš 2005). Those species of Brassicaceae analyzed to date with FC have shown small nuclear DNA contents and a narrow range of variation (Bennett and Leitch 2005). Genome size can now be superimposed over gene marker-based phylogenies for assessing ancestral DNA content, and it has provided the ability to trace genome size evolution (Marhold and Lihová 2006). Likewise, DNA content in polyploids of recent origin is expected to be proportional to the ploidy level for the purposes of assessing ploidy level (Bennett et al. 2000). This pattern is reported in several species of Draba L., which

included diploids, triploids, tetraploids, and hexaploids to 16-ploid, and this evidence was supported by molecular markers (Grundt et al. 2005; Jordon-Thaden and Koch 2008).

Polyploidization and hybridization often result in a reticulate pattern of evolution (Marhold and Lihová 2006). These events make reconstruction of evolutionary relationships challenging. Reticulate evolution can be detected by incongruences between phylogenetic trees derived from plastidic (cp) and nuclear (nr) DNA. These incongruences have been shown to be common in Lepidium between cpDNA and nrDNA ITS (internal transcribed spacers) markers, with common polyploids suggesting allopolyploid speciation in the genus (Mummenhoff et al. 2001, 2004). It has been suggested that increases or decreases in ploidy levels as determined from genome size can be useful for indicating the contributions of different parental origins (Wendel et al. 2002; Kellogg and Bennetzen 2004; Leitch and Bennett 2004).

As part of a taxonomic evaluation of native species of Lepidium from the Intermountain Western United States, a series of research efforts has been undertaken. Recent taxonomic treatments mention that this group needs further molecular studies to understand species concepts and taxonomic rankings used in the group (Holmgren 2005, Al-Shehbaz and Gaskin 2010). As part of those efforts, this survey reports on ploidy-level studies using flow cytometry methods. The morphological differences that distinguish species and infraspecific taxa within this geographic region are faint but consistent within taxa, implying possible polyploidy, reticulate evolution, or recent hybrid origins. These differences have led to uncertainty about the taxonomic arrangement of the group under review, and particularly what constitutes the species level and how these taxa are related to each other (Hitchcock 1936, 1950, Rollins 1993; Holmgren 2005; Al-Shehbaz and Gaskin 2010).

Ploidy-level information derived from flow cytometry methods is used in this study to evaluate whether or not ploidy-level patterns are helpful in revealing a taxonomic interpretation of Intermountain West Lepidium. One aspect evaluated was whether or not there are ploidy-level patterns found along geographic ranges associated with landscape or distribution patterns as reported in the literature (Suda et al. 2007; Whittemore and Olson 2011). In this study, two main questions were evaluated for developing further understanding for the classification of western

Lepidium. First, are there ploidy-level occurrences associated with geologic, geographic, or ecological settings in the taxonomically problematic Lepidium? Second, are there ploidy levels associated with certain species groups or certain infraspecific taxa within a species?

METHODS

Plant Material

Seeds were collected in the field and plants were grown in a greenhouse to provide green leaf tissue for flow cytometry (FC) (Table 2.1, Fig. 2.1). Seeds were obtained during several extensive field trips between 2007 and 2009 throughout the Intermountain West. Search locations of Lepidium of interest were developed using location data from herbarium specimens at the Gray Herbarium (GH) and from the New York Botanical Gardens Virtual Herbarium (Thiers, continuously updated) and Calflora (2008) herbaria databases online. Other voucher collections, including seeds, were collected by several botanists and shipped to the University of Alaska-Fairbanks (UAF) (Table 2.1).

Seeds were collected from three to five individuals per population. Leaf materials were collected from the same individuals for DNA extraction, and voucher specimens were collected from the population for morphology analysis and taxonomic identification.

Geographic patterns of western Lepidium were evaluated by developing distribution maps of data collection points using a geographic information system (GIS). Geographic Universal Transverse Mercator (UTM) coordinates were assigned to all Lepidium voucher specimens and displayed as point locations in ArcGIS 10.0. Based on the species localities, a distribution map was then created and digitized.

Due to uncertainty of the taxonomic status of various rankings, the nomenclature follows several sources. These include Rollins (1993), Hitchcock (1950), Holmgren (2005), and Al-Shehbaz and Gaskin (2010).

Flow Cytometry

Live leaf materials were collected from greenhouse-grown Lepidium and kept on ice until processed, which generally occurred within 1–3 hours of leaf collection. Leaves were placed in a petri dish with equal amounts of leaves from the size standard (Glycine max 'Polanka') maintained in a growth chamber at UAF. Leaf material was chopped in 0.5 mL of cold chopping buffer using a stainless-steel razor blade. The chopping buffer was modified from Otto (1990) Buffer I by adding 0.5% v/v of Tritonx100 rather than Tween 20. As the leaves were chopped, an additional 0.5 mL of cold chopping buffer was added. The samples were filtered through a 30-μm Partec CellTrics© filter and centrifuged for 20 seconds at 3500 rpm. The supernatant was drawn off, and 2 μL of RNase was added to the pellet. The pellet was resuspended in 0.2 mL of propidium iodine staining buffer (28.65 g dibasic sodium phosphate, 50 mg propidium iodine, 200 mL deionized water). Samples were stained in the dark for 40 minutes prior to flow cytometry application.

Flow cytometry (FC) was performed on a BD Bioscience FACSAria flow cytometer (BD Bioscience, San Jose, CA) equipped with FACSDiva Software (BD Bioscience, San Jose, CA). Samples were run until 10,000 nuclei were scored. The DNA content was calculated by comparing the mean peak fluorescence with the internal reference standard, Glycine max (L.) Merr. 'Polanka' (2C = 2.5 pg DNA) (Doležel and Bartoš 2005) (Fig 2.1). Following Doležel and Bartoš (2005), the 2C value of each sample (somatic cell nuclear content) was estimated as the sample peak mean/standard peak mean × standard 2C DNA content (2.5 pg DNA). To verify the range of DNA content, C values were compared to reported values on the Kew Royal Botanical Gardens DNA C-values database (Bennett and Leitch 2012). The 2C values were used to determine diploids and tetraploids by comparison to reported polyploidy levels from published chromosome counts (Warwick and Al-Shehbaz 2006).

RESULTS

Fifty-eight samples of Lepidium individuals representing 14 species and 11 varieties were surveyed (Table 2.1). Of these 58 samples, 52, or 90%, were tetraploids. Chromosome counts for six of the taxa surveyed here have been previously reported in the literature. These are L.

dictyotum A. Gray (2n = 32) (Mummenhoff et al. 2004), L. latifolium L. (2n = 24), L. lasiocarpum Nutt. ex Torrey & Gray (2n = 32), L. integrifolium Nutt. ex Torrey & Gray var. heterophyllum S. Wats (2n = 16), L. montanum Nutt. ex Torrey & Gray var. canescens (Thell.) C. L. Hitchc. (2n = 32), L. montanum var. jonesii (Rydb.) C. L. Hitchc. (2n = 32), and L. montanum var. montanum (n = 16) (Warwick and Al-Shehbaz 2006). The remainder of the taxa surveyed here is reported for the first time. For the other three taxa in this survey that were not tetraploids, the range of ploidy levels included diploid and hexaploid. Taxa having other than a tetraploid count included L. integrifolium (hexaploid), L. montanum var. canescens (diploid), and L. montanum var. cinereum (C.L. Hitchc.) Rollins (syn. var. stellae Welsh & Reveal) (hexaploid).

The distributions of ploidy levels across species surveyed are shown in Fig. 2.2. The variation of DNA content (2C value) of the species surveyed shows the overwhelming occurrence of tetraploids throughout the group of 14 species and 11 varieties sampled.

Occurrences of various ploidy levels were scattered across the Intermountain West. Based on the 58 samples surveyed, there are no obvious patterns of ploidy levels associated with landscape type, geographic range, or species complex (Fig 2.3). Though there are differences in microhabitat preferences among most of the taxa, ploidy levels were not found to be associated with any particular landscape feature. It appears that ploidy levels, besides tetraploids, vary randomly and are not influenced by landscape, habitat, or geographic isolation. Though the amount of DNA content may vary between species and within infraspecific taxa, there are generally no differences found in ploidy level between a species and its varieties except for L. montanum var. cinereum, which was found to have both tetraploid and hexaploid counts.

DISCUSSION

With the high abundance and widespread occurrence of polyploids, especially tetraploids, in the Lepidium surveyed from the Intermountain West, ploidy level does not provide much insight for explaining morphological differences or geographic patterns. However, there are a few

noteworthy observations. Ploidy levels in Lepidium as a whole show that it is not unusual for a single taxon to have more than one ploidy level. Examples of species with more than one ploidy level are L. latifolium (diploid and hexaploid), L. integrifolium (tetraploid, hexaploid, and octoploid), L. montanum var. canescens (diploid and tetraploid), L. montanum var. jonesii [diploid (Warwick and Al-Shehbaz 2006) and tetraploid], and L. thurberi (diploid and tetraploid). In the L. montanum complex, of the seven varieties surveyed, only var. cinereum had more than one ploidy level. Variety cinereum is reported as both tetraploid and hexaploid. The hexaploid is from a collection of var. stellae, which has been submerged into the concept of var. cinereum (Al-Shehbaz and Gaskin 2010).

Historically, the use of chromosome numbers for classification purposes in vascular plant taxonomy has long been limited to supporting or defining the concept of a species. The shape and number of chromosomes are not weighed differently than any other morphological feature for taxonomic purposes (Stacy 1980). This is probably a result of the frequent variation in chromosome or ploidy levels. The lack of ploidy level used to support species concepts is no doubt linked to the fact that over 70% of angiosperm plants have ploidy level increases in their evolutionary histories (Meyers and Levin 2006). With various ploidy levels being common in most angiosperm groups, it would appear that little distinction could be obtained from the variability.

Stebbins (1971) proposed that a young polyploid complex would contain many diploids and a few tetraploids. As the complex ages, more species would become polyploids, and higher ploidy levels would develop. As time progresses, the ancestor diploids would ultimately die out, resulting in it being more difficult to describe evolutionary ancestry. Almost all species in this survey were of higher ploidy levels, with 90% being tetraploids. In addition, the eleven native North American species of Lepidium with reported chromosome counts were 90% dominated by tetraploids (Warwick and Al-Shehbaz 2006). Using Stebbins's widely accepted concept (Meyers and Levin 2006), the Intermountain West Lepidium complex can be described as an evolutionarily young to moderate-aged species group with a reticulate evolutionary past. The indication of a reticulate evolutionary past has been demonstrated in Lepidium by incongruences in gene trees (Bowman et al. 1999, Mummenhoff et al. 2001, 2004). Bowman et al. (1999) and

Mummenhoff et al. (2001, 2004) suggested that most polyploids in Lepidium are allopolyploid in the genus. On the basis of previous cpDNA and ITS sequence data, allopolyploidization has been strongly favored to explain gene tree patterns (Mummenhoff et al. 2001, 2004). The influence of a reticulate evolutionary past in the Intermountain West region in this genus, coupled with a high occurrence of polyploidy across all species and with highly mobile seeds that are easily transported by avian species to allow for mixing of populations and species (Al-Shehbaz 1986), provides for a diverse array of polyploidy genotypes (Soltis and Soltis 1999). This evolutionary background may be setting the challenging stage for adequately classifying these native Intermountain species.

In future research on Intermountain West Lepidium, DNA sequences will be analyzed and gene trees developed. Without gene trees, these FC observations and indications of input of different parental DNA stock based on variable sizes of DNA content cannot be conclusively resolved. Likewise, with no patterns in geographic ploidy levels associated with geographic ranges or habitats and with variability of DNA content between Lepidium taxa, mere ploidy levels may not be useful for taxonomic purposes, but they do indicate the need for gene trees to be constructed to adequately treat these diverse native species of Lepidium in the Intermountain West.

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CHAPTER 2 TABLES

Table 2.1. Species, collection locations, 2C values, and ploidy levels.

Species	Voucher Number	Location	2C	Inferred Ploidy Level	Species Reported Previously in Literature
L. alyssoides A. Gray var. alyssoides	10502	Big Piney, WY	1.619	Tetraploid, 2n = 32	Rollins (1993), n = 16
	8443	Sweetwater Canyon, NM	1.526	Tetraploid, 2n = 32	
	8519	Dinosaur National Monument, CO	1.738	Tetraploid, 2n = 32	
	8519c		1.451	Tetraploid, 2n = 32	
L. alyssoides var. eastwoodiae (Wooton) Rollins	10624 (f/d)	Hay Gulch, CO	1.699	Tetraploid, 2n = 32	
	8909a	White Sands National Monument, NM	1.357	Tetraploid, 2n = 32	
	8509b		1.346	Tetraploid, 2n = 32	
	8909c2		1.327	Tetraploid, 2n = 32	
	10624	Hay Gulch, CO	1.444	Tetraploid, 2n = 32	

Species	Voucher Number	Location	2C	Inferred Ploidy Level	Species Reported Previously in Literature
L. barnebyanun Reveal	8520	Duchesne, UT	1.551	Tetraploid, 2n = 32	
L. crenatum (E. L. Greene)	8518	Hamilton, CO	1.603	Tetraploid, 2n = 32	
	8518a2		1.384	Tetraploid, 2n = 32	
	8518b3		1.402	Tetraploid, 2n = 32	
L. davisii Rollins	8525	North rim of Snake River, ID	1.506	Tetraploid, 2n = 32	
	8525		1.38	Tetraploid, 2n = 32	
L. dictyotum A. Gray	8527	North of Sparks, NV	1.228	Tetraploid, 2n = 32	Mummenhoff et al. (2004), 2n = 32
L. fremontii S. Wats. var. stipulata	8528b	Buffalo Well, NV	1.229	Tetraploid, 2n = 32	
	8528		1.458	Tetraploid, 2n = 32	
L. huberi S. L. Welsh & Goodrich	10465	Wamsutter, WY	1.75	Tetraploid, 2n = 32	
	10456	Vernal, UT	1.555	Tetraploid, 2n = 32	

Species	Voucher Number	Location	2C	Inferred Ploidy Level	Species Reported Previously in Literature
L. integrifolium Nutt. ex Torrey & Gray	8522	Bear River Wildlife Area, WY	2.001	Tetraploid, 2n = 32	
	8522		3.117	Hexaploid, 2n = 64	
	10452	Cokeville, WY	1.307	Tetraploid, 2n = 32	
L. integrifolium var. heterophyllum S. Wats.	24083	Cedar City, UT	1.239	Tetraploid, 2n = 32	
L. latifolium L.	8534	Ely, NV	0.892	Diploid, 2n = 16	Mulligan (1957), 2n = 24; Queirós (1973), 2n = 24; Montgomery et al. (1997), 2n = 24. For others, see Warwick and Al-Shehbaz (2006) Queirós (1979), 2n = 48
	8534		0.768	Diploid, 2n = 16	

Species	Voucher Number	Location	2C	Inferred Ploidy Level	Species Reported Previously in Literature
L. montanum var. canescens	8530	Battle Mt., UT	1.587	Tetraploid, 2n = 32	Rollins and Rüdenberg (1971), 2n = 32
	8529	Paradise Valley, UT	1.613	Tetraploid, 2n = 32	
	8523a	Hollbrook Spring, NV	0.869	Diploid, 2n = 16	
	8523c2		0.929	Diploid, 2n = 16	
L. montanum var. cinereum	24136	Fredonia, AZ	3.165	Hexaploid, 2n = 64	
	8524a3	Hollbrook Ranch, NV	1.533	Tetraploid, 2n = 32	
	8524b		1.516	Tetraploid, 2n = 32	
	8524c1		1.537	Tetraploid, 2n = 32	
L. montanum var. coloradense Rollins	8517a	Gypsum, CO	1.555	Tetraploid, 2n = 32	
	8517b		1.439	Tetraploid, 2n = 32	
	8517c		1.444	Tetraploid, 2n = 32	

Species	Voucher Number	Location	2C	Inferred Ploidy Level	Species Reported Previously in Literature
L. montanum var. jonesii (Rydb.) C. L. Hitchc.	8537	Vernal, UT	1.486	Tetraploid, 2n = 32	Schaack et al. (1984), n = 16; Rollins and Rüdenberg (1971), n = 16; Rollins and Rüdenberg (1977), n = 16; Rollins (1993), 2n = 32
	8521c		1.219	Tetraploid, 2n = 32	
	8521c		1.479	Tetraploid, 2n = 32	
L. montanum var. montanum	8532	Twin Bridges along Huntington Creek, NV	1.565	Tetraploid, 2n = 32	Weedin and Powell (1978), n = 16; Rollins (1993), n = 16
	8531	Whirlwind Valley, NV	1.516	Tetraploid, 2n = 32	
	8516c3	Powderhorn, CO	1.419	Tetraploid, 2n = 32	
	8516c3		1.347	Tetraploid, 2n = 32	

Species	Voucher Number	Location	2C	Inferred Ploidy Level	Species Reported Previously in Literature
L. montanum var. tenellum (L. O. Williams) C. L. Hitchc.	71608	Mesa City, CO	1.271	Tetraploid, 2n = 32	
			2.044	Tetraploid, 2n = 32	
			1.895	Tetraploid, 2n = 32	
			2.123	Tetraploid, 2n = 32	
L. montanum var. wyomingense (C. L. Hitchc.) C. L. Hitchc.	10500	Lone Tree, WY	1.606	Tetraploid, 2n = 32	
L. nanum S. Wats.	8533	Jake's Valley, NV	1.4	Tetraploid, 2n = 32	
L. ostleri Welsh & Goodrich	24080	San Francisco Mts, UT	0.908	Diploid, 2n = 16	

Species	Voucher Number	Location	2C	Inferred Ploidy Level	Species Reported Previously in Literature
L. thurberii Wooten	8511 dry	Lordsburg, NM	0.631	Diploid, 2n = 16	
	8510a	Columbia, NM	0.699	Diploid, 2n = 16	
	8511a	Lordsburg, NM	1.319	Tetraploid, 2n = 32	
	8511b1		1.316	Tetraploid, 2n = 32	
	8511c		1.354	Tetraploid, 2n = 32	
L. tiehmii (Rollins) Al-Shehbaz (syn. Stroganowia tiehmii Rollins)	10524	Table Mountain, NV	1.658	Tetraploid, 2n = 32	

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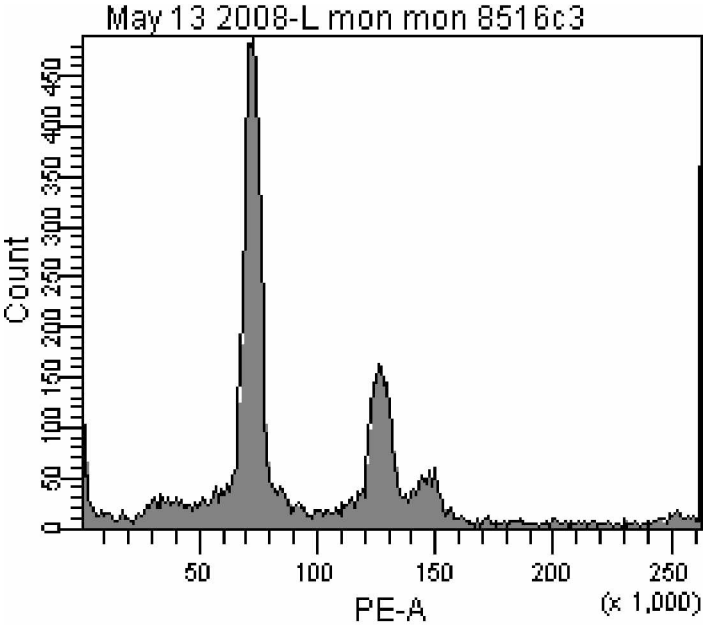

Figure 2.1. Histogram showing Phycoerythrin-A (PE-A) values for Glycine max standard at 75,000 and a sample of tetraploid L. montanum Nutt. var. montanum at about 140,000 absorption units.

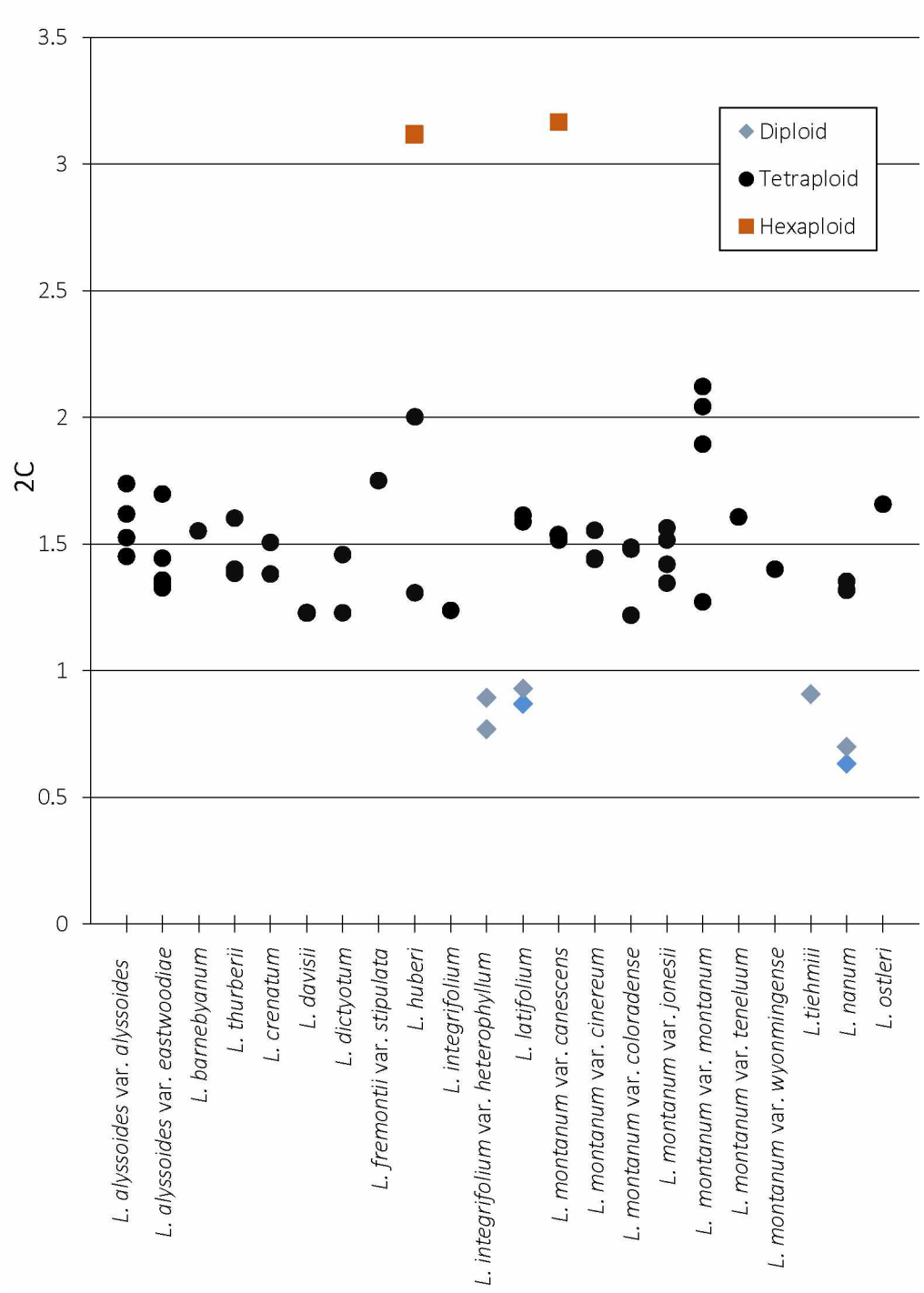

Figure 2.2. Distribution of DNA content (2C) ranges across all species surveyed.

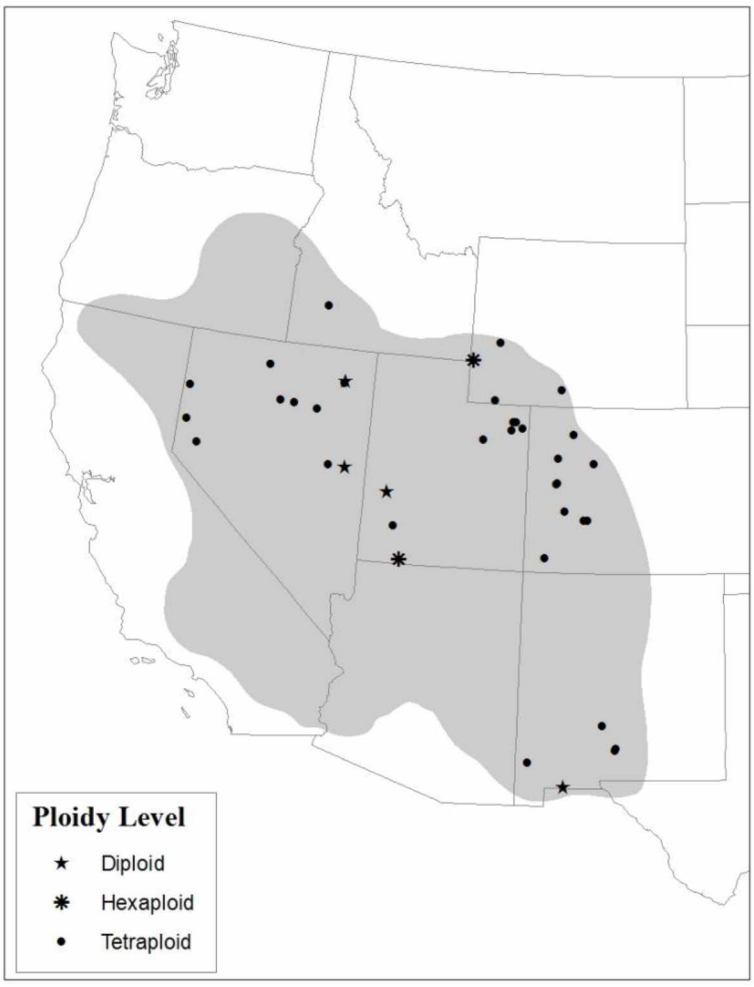

Figure 2.3. Distribution of Lepidium with various ploidy levels in the Intermountain West.

CHAPTER 3: PHYLOGENETIC SURVEY OF THE INTERMOUNTAIN WEST MEMBERS OF THE GENUS LEPIDIUM L. (BRASSICACEAE) IN THE UNITED STATES

ABSTRACT

The taxonomic status of members of the genus Lepidium in the Intermountain West has been in flux for years. Species concepts and classification of these endemic species from the western United States centers on the highly variable L. montanum complex. Until recently, classification treatments using morphological features in this group have been adequate, but as more new species are discovered and more locations reported, the limited number of morphological features available for classification and defining species concepts has led to more uncertainty about taxonomic rankings. As part of a molecular and morphological-based treatment of the group, sequences of both nuclear ribosomal (internal transcribed spacers, (ITS1, ITS2)) were produced for 59 samples of Intermountain Lepidium from across the region including the west coast of the United States. Results were difficult to interpret and the patterns manifest several known issues in using ITS as a single marker in closely related species. In summary, phylogenetic resolution was poor, regardless of which samples were included. The results provide insight into this radiant speciation group of Intermountain West Lepidium. The closely related samples focus where future efforts might resolve many of the taxonomic questions about the rankings of taxa and validity of many of the varieties included in L. montanum.

INTRODUCTION

Brassicaceae are distributed worldwide and consist of 49 tribes, 321 genera, and more than 3660 species (Al-Shehbaz 2012). The family has been subject to many studies using molecular approaches in recent years (Bailey et al., 2006; Beilstein et al., 2006, 2008; Al Shehbaz et al. 2006; Warwick et al., 2010; Al-Shehbaz, 2012). Based on phylogenetic inference, these studies have provided the basis for understanding the relationships among various taxonomic components within the family and patterns of morphological development and their uses in classification. Within the family, the most common genetic markers used have been transcribed nuclear ribosomal spacer (ITS), plastid DNA (cpDNA), and nuclear ribosomal DNA (rDNA)

because these markers tend to be highly conserved sequences with high copy numbers, making them easily accessible without molecular cloning.

ITS regions have been used extensively within the Brassicaceae to help test various treatments of taxonomic classification. Clarification of tribal arrangements using ITS has a long history within the family (Warwick and Black 1994; Crespo et al. 2000; Koch (2003); Koch et al. (2003a); Mitchell-Olds et al. (2005); Beilstein et al. 2006; Mummenhoff et al. 2001a, 2005), between genera within tribes (Warick, et al. 2009, 2010; German et al. 2009) and across species-level studies (Franzke and Mummenhoff 1999 Koch et al. 2003; Smith et al. 2009). ITS has been used as a single gene marker in some studies and coupled with chloroplast markers in many other studies. The limitations of ITS for phylogenetic studies have been discussed by various authors, and specific issues have been identified that limit its use (Poczai and Hyvönen 2010). Some of the issues include the fact that ITS is not a single locus, but rather copies are spread across the genome. In most cases, sequence identity is maintained across copies of ITS via concerted evolution (Poczai and Hyvönen 2010). However, for studies of closely related species or in groups with highly radiant speciation, limited concerted evolution and sequence divergence can confound inferences (Sanderson and Doyle 1992).

As part of an ongoing taxonomic evaluation of native species of Lepidium from the Intermountain West of the United States, I have undertaken a series of research efforts. Recent taxonomic treatments mention that the Intermountain group needs further molecular studies to clarify species concepts and taxonomic rankings (Holmgren 2005; Smith, et al. 2009; Al-Shehbaz and Gaskin 2010). As part of those efforts, Lichvar (2015) reported ploidy levels for 14 species and 11 varieties from across the western United States. Those efforts were in support of evaluating the possible reasons for the faint morphological differences that distinguish species and infraspecific taxa within this geographic region, implying possible polyploidy, reticulate evolution, or recent hybrid origins (Stebbins 1971). These differences have led to uncertainty about the taxonomic arrangement of the group under review, particularly about what constitutes the species level and how these taxa are related to each other (Hitchcock 1936, 1950, Rollins 1993; Holmgren et al. 2005; Al-Shehbaz and Gaskin 2010).

Ploidy levels for 58 samples representing 14 species and 11 varieties of Intermountain Lepidium taxa surveyed found that 90% were tetraploid, and several were diploid and hexaploid (Lichvar 2015). These ploidy level observations, and differences of c-value sizes, indicated possible input of different parental genomes. The results of ploidy levels were not aligned with any morphological features or taxonomic treatment within the group. Likewise, there was no association between ploidy levels and geographic ranges or habitats, and DNA content among Lepidium taxa did not appear to be useful for taxonomic purposes. However, ploidy levels did indicate the need for the construction of gene trees to adequately treat these diverse native species of Lepidium in the Intermountain West.

Polyploidization and hybridization often result in a reticulate pattern of evolution (Marhold and Lihová 2006). These events make reconstruction of evolutionary relationships challenging. Reticulate evolution can be detected by incongruences between phylogenetic trees derived from plastidic (cp) and nuclear (nr) DNA or by comparing nuclear loci. These incongruences are common in Lepidium between cpDNA and nrDNA and ITS markers, with common polyploids suggesting allopolyploid speciation in the genus (Mummenhoff et al. 2001, 2004).
My objective here is to provide a phylogenetic understanding of the relationships among the Intermountain Lepidium. Central to the Intermountain group of taxa under consideration is the L. montanum group (Table 1). The complexity and variations of the morphology in this group are still unclear as to the most accurate treatment of species and infraspecific taxa. The recognition of seven of Hitchcock's (1936, 1950) infraspecific taxa as distinct species reduced the heterogeneity in Lepidium montanum (Rollins 1993; Holmgren 2005). Even the remaining 12 varieties accepted in the species only partially cover its overall complexity and relationships between each of the taxa (Hitchcock 1936, 1950, Rollins 1993; Holmgren 2005; Al-Shehbaz and Gaskin 2010). In an effort to bring clarity to the Intermountain group of Lepidium, I undertook a molecular ITS evaluation for the purpose of determining taxonomic clarity in this group.

METHODS

Sequences of both nuclear ribosomal (internal transcribed spacers, (ITS1, ITS2)) were produced for 59 samples (Table 3.1) of Intermountain Lepidium from across the region including the west

coast of the United States. Nomenclature in Table 3.1 follows Al-Shebaz and Gaskin (2010) for most species but follows Rollins (1993) for all the varietal treatments of L. montanum. The majority of these were collected by the author and several volunteer collectors. A total of 44 taxa were collected in the field across the Intermountain West and west coast, mainly in California. Collections included DNA samples and vouchers. Vouchers are deposited in the Intermountain Herbarium, Logan UT and at the Alaskan Science Museum herbarium in Fairbanks, AK. Methods for DNA extraction, polymerase chain reaction and direct sequencing of ITS are given elsewhere (Bowman et al. 1999; Mummenhoff et al. 2001). From the 382 samples representing the 44 taxa, 764 ITS sequences were generated and cleaned.

Consensus sequences were built by using make.consensus.seqs functions in sangeranalyseR (https://github.com/roblanf/sangeranalyseR). The following settings were used for read cleaning: min.reads = 2, trim.cutoff = 1e-04, min.length = 20, max.secondary.peaks = NULL, secondary.peak.ratio = 0.33, minInformation = 0.5, threshold = 0.5 (sangeranalyseR::make.consensus.seqs). Low quality sequences and most secondary peaks in the chromatograms were removed. Because of the high numbers of secondary peaks, very few individuals had enough data. Using a trim.cutoff (= 0.0001) left 238 samples in the alignment (cs290.html). With all the restrictions applied to improve analysis capabilities the following analyses were done; a) all samples, b) all samples including outgroup species of Lepidium species from California, and c) only samples from the intermountain and rocky mountain regions. The ITS contig length variation in the samples was extensive (Fig. 3.1).

Next, the dealt sequence lengths usable for phylogenetic were analyzed. We attempted to select sequences that were sufficiently long to proceed to phylogenetic analysis. Our samples had fewer sequences long enough for analysis, which would limit the number of samples that could be analyzed (Fig. 3.2). To accommodate the variability in sequence length, the following cutoff values were used: 0.3, 0.5, and 0.7. Of the 238 samples, this retained 200, 152, and 130 samples in the analyses. The most useful output was the analysis using 130 samples representing 27 taxa.

RESULTS

Results herein are difficult to interpret and the patterns manifest several known issues in using ITS as a single marker in closely related species. Feliner and Rosellio (2007) expressed concern that non-cloned ITS sequences retrieved from PCR product share many of the same target priming sites in one or more loci which can cause consensus ITS sequences. So, when non-cloned ITS sequences are used as raw data in reconstruction of phylogenetic relationships, it acts as a molecular phenotype from which the genotype can't always be inferred. Other drawbacks of ITS data include that there are hundreds or thousands of ITS copies spread across the genome (Alvarez and Wendel 2003). Inferring phylogenies from ITS and multigene families can lead to incorrect results because there is variation within the different repeats in the genome (Coleman 2003). Buckler et al. (1997) provided evidence that the variation among ITS sequences occurs mainly in hybrids and polyploids. These basic points about the variation within ITS sequences across the genome then with concerted evolution make phylogenetic interpretations even more difficult (Poczai and Hyvönen 2010), in turn leading to different classes of ITS (Buckler et al. 1997, Wendel et al. 1995, Soltis and Soltis 1991, and Ritland et al. 1993). These differences from concerted evolution, hybridization and polyploidy, and repeats of ITS with different gene families, can cause intra-individual paralogues among sequences of an individual and can be maintained and shared with other species causing problems in phylogenetic analysis.

The ITS phylogenetic tree developed from this study (Fig. 3.3) amplifies many of the issues of using ITS for closely related species. A modified and reduced version of Mummenhoff et al. (2001) ITS global survey of the genus shows west coast (California) of the United States as a different clade than the western with its intermountain clade (Fig. 3.1) with L. montanum from the intermountain region anchored with this separate clade.

As the outgroup in this study, the species from the west coast of the United States species in the California clade sorted separately as a clade and also as our outgroup in our ITS tree. The diploid taxa from Lichvar (2015) of L. ostleri, L. thurberi, and L. montanum var. canescens showed weak associations of tightly clustered clades nearer to the root of the tree. But samples of these taxa also have scattered occurrences in other parts of the tree. In part, the ITS sequences of the

diploids tended to show relationships with samples from the southern and western reaches of the study samples (Fig. 3.4).

Geographic patterns of inferred sequences for related taxa was faint for several groups (Fig. 3.5). For example, L. heterophyllum more typically located in the Oregon, Washington and Idaho region has an outlier population near Durango, CO with overlapping geographic ranges with L. montanum var. neeseae, L. montanum var. nevadensis, and L. crenatum and other L. montanum varieties. But these overlapping distribution patterns are weak and no inferences other than a faint geographic overlap ranges associated with most of the L. montanum varieties can be detected. Smith et al. (2009) using various gene markers discussed east and west clades of patterns in the taxa of Lepidium they surveyed. Their samples were limited to a smaller region of the Intermountain West than the broader range of samples surveyed here. Those patterns may be less meaningful when more of the intermountain taxa are included in an analysis.

Another pattern observed in this study is the proximity of caespitose entire leafed species generated in the ITS tree. The genus has the following narrow endemics with mostly linear entire basal leaves, as L. ostleri, L. barnebyanum, L. nanum and L. davisii. These species are not sympatric in distribution nor do they occur in similar habitats. L. davisii is found on clay vernal playas in parts of ID and NV. L. ostleri is found on limestone gravel opening in ponderosa pine forest in Utah while L. barnebyanum is located on white shale ridges in Utah. Interestingly these three species and also L. nanum are generally all caespitose with entire leaves that tend to be slightly fleshy. This is a distinct growth form in the genus and in the ITS tree in this study, these taxa usually are in the same clade or even closely related branch tips. Though the inferences from the ITS analysis are weak, they indicate a possible relationship between these species.

In the Intermountain West, the species that is most commonly associated with all clades and branches is L. montanum. This species starts to make its appearance near the root of the tree and is scattered throughout all branches and clades. This taxon has a relationship with all the other species and varieties in this region which suggests introgression into many related taxa.

DISCUSSION

Rapid and radiant speciation in the Brassicaceae evolution, as pointed out by Franzke et al. (2011), is supported by a strong phylogenetic congruence when based on nuclear rDNA ITS (Bailey et al. 2006), chloroplast ndhF (Al-Shehbaz et al.2006) and nuclear phyA (Beilstein et al. 2008) sequence data in the basal part of the family. Alternatively, the lack of some basal resolution in phylogenetic trees could be the product of "among-tree conflict," possibly caused by processes such as recombination or ancient hybridization (Franzke et al. (2011). Within the family, genetic clarity between tribes, genera and species is a common issue. Rapid expansions of genera and species in the Brassicaceae is common within many species groups including Lepidium. To evaluate relationships and species concepts within the Intermountain Lepidium, a variety of both morphological and molecular data were used.

The most problematic section within Lepidium in the United States is the Intermountain group, particularly the L. montanum group. In Chapter 1, when using morphological traits, the Intermountain species sorted into reasonable morphologically-based clades of sister species using a discrete character parsimony algorithm (PARS) (Felstenstein, Univ. Washington; http://evolution.genetics.washington.edu/phylip/doc/pars.html). But in treating western U.S. species, both Rollins (1993) and Holmgren et al. (2005) remarked about the lack of understanding for the relationships that exist between L. barnebyanum and L. davisii, L. papilliferum and L. montanum, and the treatment of L. huberi through the L. alyssoides branch of species. However, all of these species cluster nicely when based on morphological features. Statements about relationships between select species made by Rollins (1993) and Holmgren et al. (2005) are reinforced when noting the incongruency between the cladogram and the preliminary nuclear ITS results in this study (Chapter 1). These clearly indicate that some of the species noted by these authors were more closely related to other species as identified using phylogenetic approaches.

In three phylogenetic trees using different genes, there are incongruences between several clades. The ITS phylogenetic relationships of seven western U.S. species sorted into two separate clades (Fig. 3.1). Clade C, labeled California, contains four species with distribution patterns mainly

from the west coast of the U.S. and into Nevada. Clade B was a mix of California and other western U.S. species that aligned with more widespread and weedy species found throughout North America. In Clade B, L. flavum Torr. splits at an equal node that sorts sister species L. fremontii S. Watson and L. montanum Nutt. The cpDNA positioned L. fremontii, L. montanum, and L. flavum together in the same final branch in clade A (Fig. 3.2). Similar to ITS, cpDNA placed these three western U.S. species closer to more widespread species from across North America. Eurasian species also linked phylogenetically to North American Lepidium (Mummenhoff et al. 2001) in Clade A, including both western U.S. and more widespread North American species. The California-restricted Lepidium aligned in their own sub-clade. In ITS studies, the Eurasian species were a separate clade (Fig. 1.1). While Pistillata analysis (PI) shows some similar alignments it does divide the western species into two clades. In the PI phylogenetic alignment, western species of L. fremontii, L. montanum, and L. flavum were located in both major Clades A and C (Fig. 1.3). Again, L. montanum and L. flavum aligned with more widespread Lepidium of Clade A from North America. Likewise, L. fremontii and L. montanum aligned with other widespread Lepidium in the other major Clades C and D. Interestingly, L. montanum occurred twice, once in Clade A with L. flavum and again in Clade C with L. fremontii. The Eurasian species L. lyratum L. aligned with the western U.S. and widespread Lepidium of Clades A and B.

Typically species with faint and weak morphological features that are difficult to tell taxa apart many times are a result of reticulate evolution or hybridization (Linder and Rieseberg 2004). Since the Intermountain Lepidium have both weak traits for distinguishing species, hybridization was suspected and ploidy levels were surveyed (Chapter 2). With the high abundance and widespread occurrence of polyploids, especially tetraploids, in the Lepidium surveyed from the Intermountain West, ploidy levels do not provide much insight for explaining morphological differences or geographic patterns. However, there are a few noteworthy observations.

Ploidy levels in Lepidium as a whole show that they are not unusual for a single taxon to have more than one ploidy level. Examples of species with more than one ploidy level are L. latifolium (diploid and hexaploid), L. integrifolium (tetraploid, hexaploid, and octoploid), L. montanum var. canescens (diploid and tetraploid), L. montanum var. jonesii (diploid

[Warwick and Al-Shehbaz 2006] and tetraploid), and L. thurberi (diploid and tetraploid). Of the seven varieties surveyed in the L. montanum complex, only var. cinereum and var. canescens had more than one ploidy level. Variety cinereum is reported as both tetraploid and hexaploid, and var. canescens as both diploid and tetraploid. The hexaploid reported for var. cinereum is from a collection of var. stellae, submerged into the concept of var. cinereum (Al-Shehbaz and Gaskin 2010). However, it is important to note that the results of this survey may not support a significant inference about the lack of correlation between ploidy level and taxonomic, geographic, or habitat group because the number of non-tetraploid species was so small. Historically, the use of chromosome numbers for classification purposes in vascular plant taxonomy has long been limited to supporting or defining the concept of a species. The shape and number of chromosomes are not weighed differently than any other morphological feature for taxonomic purposes. The lack of use of ploidy level to support species concepts is no doubt linked to the fact that over 70% of angiosperm plants have ploidy level increases in their evolutionary histories (Meyers and Levin 2006). Because multiple ploidy levels are common in most angiosperm groups, it would appear that little distinction among taxa could be obtained from the variability. Stebbins (1971) proposed that a young polyploid complex would contain many diploids and a few tetraploids. As the complex ages, more species would become polyploids and higher ploidy levels would develop. As time progresses, the ancestor diploids would ultimately die out, resulting in greater difficulty in describing their evolutionary ancestry. Almost all species in this survey were of higher ploidy levels, 90% being tetraploids. In addition, the 11 native North American species of Lepidium with reported chromosome counts were 90% dominated by tetraploids (Warwick and Al-Shehbaz 2006). Using Stebbins's widely accepted concept (Meyers and Levin 2006), the Intermountain West Lepidium complex can be described as an evolutionarily young to moderately-aged species group with a reticulate evolutionary past. The indication of a reticulate evolutionary past has been demonstrated in Lepidium by incongruences in gene trees (Bowman et al. 1999; Mummenhoff et al. 2001, 2004). Bowman et al. (1999) and Mummenhoff et al. (2001, 2004) suggest that most of the species that are polyploids in Lepidium are allopolyploid. On the basis of previous cpDNA and ITS sequence data, allopolyploidization has been strongly favored to explain gene tree patterns (Mummenhoff et al. 2001, 2004). The influence of a reticulate evolutionary past in the Intermountain West region in this genus, coupled with both high occurrence of polyploidy across all species and

highly mobile seeds that are easily transported by avian species to allow for mixing of populations and species (Al-Shehbaz 1986b), provides for a diverse array of polyploid genotypes (Soltis and Soltis 1991). This evolutionary background may be setting a challenging stage for adequately classifying these native intermountain species. ITS molecular research (Chapter 3) on Intermountain West Lepidium led to DNA sequences that were analyzed and gene trees developed. Without gene trees, these flow cytometry observations and indications of input of different parental DNA stock based on variable sizes of DNA content cannot be conclusively resolved. Likewise, with no association of geographic ploidy levels with geographic ranges or habitats and with variability of DNA content between Lepidium taxa, mere ploidy levels may not be useful for taxonomic purposes. However, ploidy levels did indicate the need for the construction of gene trees to adequately treat these diverse native species of Lepidium in the Intermountain West.

Results in Chapter 3 are difficult to interpret and the patterns manifest several known issues in using ITS as a single marker in closely related species. Feliner and Rosellio (2007) expressed concern that non-cloned ITS sequences retrieved from PCR product share many of the same target priming sites in one or more loci which can cause consensus ITS sequences. So, when non-cloned ITS sequences are used as raw data in reconstruction of phylogenetic relationships, it acts as a molecular phenotype from which the genotype can't always be inferred. Other drawbacks of ITS data include that there are hundreds or thousands of ITS copies spread across the genome (Alvarez and Wendel 2003). Inferring phylogenies from ITS and multigene families can lead to incorrect results because there is variation within the different repeats in the genome (Coleman 2003). Buckler et al. (1997) provided evidence that the variation among ITS sequences occurs mainly in hybrids and polyploids. These basic points about the variation within ITS sequences across the genome with concerted evolution make phylogenetic interpretations even more difficult (Poczai and Hyvönen 2010), in turn leading to different classes of ITS (Buckler et al. 1997; Wendel et al, 1995; Soltis and Soltis 1991; and Ritland et al. 1993). Differences from concerted evolution, hybridization and polyploidy, and repeats of ITS with different gene families, can cause intra-individual paralogues among sequences of an individual and can be maintained and shared with other species causing problems in phylogenetic analysis.

The ITS phylogenetic tree developed from this study (Fig. 3.3) amplifies many of the issues of using ITS for closely related species. A modified and reduced version of Mummenhoff et al. (2001) ITS global survey of the genus shows US west coast (California) as a different clade than the western group with its intermountain clade (Fig. 3.1) with the intermountain region L. montanum anchored with this separate clade.

As the outgroup in this study, species from the west coast of the United States in the California clade sorted separately as a clade and also as our outgroup in our ITS tree. Diploid taxa from Lichvar (2015) of L. ostleri, L. thurberi, and L. montanum var. canescens showed a weak association of tightly clustered clades nearer the root of the tree. But samples of these taxa also have scattered occurrences in other parts of the tree. In part, the ITS sequences of the diploids tended to show relationships with samples from the southern and western reaches of the study samples (Fig. 3.4).

Geographic patterns inferred from sequences for related taxa was faint for several groups (Fig. 3.5). For example, L. heterophyllum more typically located in the Oregon, Washington and Idaho region has an outlier population near Durango, CO with overlapping geographic ranges with L. montanum var. neeseae, L. montanum var. nevadensis, and L. crenatum and other L. montanum varieties. But these overlapping distribution patterns are weak and provide no inferences other than faint geographic overlapping ranges associated with most of the L. montanum varieties. Smith et al. (2009), using various gene markers, discussed eastern and western clades of patterns in taxa of Lepidium they surveyed. Their samples were limited to a smaller region of the Intermountain West than the broader range of samples surveyed here. Those patterns may be less meaningful when more of the intermountain taxa are included in an analysis.

Another pattern observed in this study is the proximity of caespitose entire leafed species generated in the ITS tree. The genus has the following narrow endemics with mostly linear entire basal leaves, as L. ostleri, L. barnebyanum, L. nanum and L. davisii. These species are not sympatric in distribution nor do they occur in similar habitats. L. davisii is found on clay vernal playas in parts of ID and NV. L. ostleri is found on limestone gravel opening in ponderosa pine

forest in Utah while L. barnebyanum is located on white shale ridges in Utah. Interestingly these three species, and also L. nanum, are generally all caespitose with entire leaves that tend to be slightly fleshy. This is a distinct growth form within the genus and in the ITS tree in our study. These taxa usually are in the same clade or even on closely related branch tips. Though inferences from the ITS analysis are weak, they indicate possible relationships between these species.

In the Intermountain West, the species that is most commonly associated with all clades and branches is L. montanum. This species starts to make its appearance near the root of the tree and is scattered throughout all branches and clades. This taxon has a relationship with all other species and varieties in this region, which suggests introgression into many related taxa.

CONCLUSIONS

Though the ITS molecular efforts did little to resolve either species relationships or a better insight to the nomenclature and taxonomy of Lepidium, several key points have been made. The use of morphological features by previous taxonomic authorities stood as a way to classify and distinguish Intermountain species of Lepidium. However, in limited ITS analysis using the same groups of species as in the morphological cladistics analysis, there were incongruences between the two approaches. The molecular analysis showed that the relationships were not similar in most cases as to the clustering of clades in the morphological approach. This comparison indicated possible frequent hybridization between many of the species. Yet, the ploidy level analysis showed no relationships within very difficult to classify taxa such as L. montanum varieties or any other morphological features or geographic patterns. Tetraploids were a common ploidy level with a few diploids and one hexaploid. But none of these ploidy levels were connected to any meaningful patterns to provide insights into hybridization causing faint and weakly distinct features useful for taxonomic purposes. And because of various issues with using one gene analysis and issues associated with ITS within the genome, no conclusive evidence was obtained to reliably observe patterns of divergence or relationships amongst Lepidium species. One possible species associated with parental stock involved the divergence of many species is L. montanum. In ITS analysis, this species is associated with each clade or

pattern of association. It is found in basal groups as well as on multiple tips of many clade branches. Understanding this species using molecular methods is key to improving the classification of Lepidium in the Intermountain West.

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CHAPTER 3 TABLES

Table 1. Vouchers and sample numbers supporting ITS analysis

Sample	Species	Collector	Location	Nomenclature
1	L. alyssoides A.Gray var alyssoides	Dorn 10502	Big Piney, Sublette Co., WY	Rollins 1993
2	L. alyssoides var. alyssoides	Dorn 10503	Marbelton, Sublette Co., WY	Rollins 1993
3	L. alyssoides var alyssoides	Rollins 1896 (GH)	Mesa Co. CO	Rollins 1993
4	L. alyssoides var alyssoides	Lichvar 8519	Dinosaur NP, Moffat Co., CO	Rollins 1993
5	L. allysoides var. angustifolium (C.L. Hitchc.) Rollis	Rollins and Corell 6185 (GH)	Brewster Co., TX	Rollins 1993
6	L. barnebyanum Reveal	Dorn 10454	Duchesne Co., UT	FNA
7	L. barnebyanum	Neese and S. Wesh 8910 (GH)	Duchesne Co., UT	FNA
8	L. crenatum (E.L. Greene) Rydb.	Rollins 1951 (GH)	Paonia, Delta Co., CO	FNA
9	L. crenatum	Lichvar 8518	Hamilton, Moffat Co., CO	FNA
10	L. crenatum	Ownbey 1462 (GH)	Montezuma Co., CO	FNA

Sample	Species	Collector	Location	Nomenclature
11	L. crenatum	Lichvar 8513	Mesa Verde NP, Montezuma Co., CO	FNA
12	L. davisii	Lichvar 8525	Mtn. Home, Elmore Co., ID	FNA
13	L. davisii Rollins	Debolt 1030 (GH)	Twin Lakes, Owybee Co., ID	FNA
14	L. dictyotum A.Gray	Eretter 8142 (GH)	Contra Costa Co., CA	FNA
15	L. eastwoodiae Wooton	Hitchock 6159 (GH)	SE of Las Vegas, Clark Co., NV	FNA
16	L. eastwoodiae	Lichvar 8509	White Sands NP, Otero Co., NM	FNA
17	L. intergrifolium Nutt. Ex Torrey & A. Gray var. integrifolium	Tiehm 15666 (GH)	Railroad Valley, Nye Co., NV	Rollins 1993
18	L. intergrifolium var. integrifolium	Lichvar 8522	S. Cokeville, Lincoln Co., WY	Rollins 1993
19	L. intergrifolium var. integrifolium	Dorn 10453	N. Evanston, Uinta Co., WY	Rollins 1993
20	L. intergrifolium var. integrifolium	Dorn 10452	S. Cokeville, Lincoln Co., WY	Rollins 1993
21	L. jaredii Brandegee	Eastwood 4116 (GH)	Soda Lake, San Luis Obispo Co., CA	FNA
22	L. lasiocarpum Nutt. Ex Torrey & Gray var. wrightii (A. Gray) Thellung	Cory (GH)	El Paso Co., TX	FNA

Sample	Species	Collector	Location	Nomenclature
23	L. latifolium L.	Dorn 10491	Burns, Harney Co., OR	FNA
24	L. montanum var. alpinum S. Wats.	Lichvar 8535	Twin Peaks, Salt Lake C., UT	Rollins 1993
25	L. montanum var. canescens	Lichvar 8533	Jake's Valley, White Pine Co., NV	Rollins 1993
26	L. montanum var, canescens (Thell.) C.L. Hitchc.	Lichvar 8529	Paradise Valley, Humbolt Co., NV	Rollins 1993
27	L. montanum var, canescens (Thell.) C.L. Hitchc.	Lichvar 8530	Battle Mtn, Lander Co., NV	Rollins 1993
28	L. montanum var. heterphyllum (Watson) C.L. Hitchoch.	Fertig 24083	Cedar City, Iron Co., UT	
29	L. montanum var. jonesii (Rydb.) C.L. Hitchc.	Dorn 10461	Browns Hole, Uintah Co., UT	Rollins 1993
30	L. montanum var. jonesii	Dorn 10459	Vernal, Uintah Co., UT	Rollins 1993
31	L. montanum var. jonesii	Dorn 10458	Jensen, Uintah Co., UT	Rollins 1993
32	L. montanum var. jonesii	Lichvar 8537	N of Vernal, Uintah Co., UT	Rollins 1993
33	L. montanum var. jonesii	Fertig 23802	Deer Creek, Garfield Co., UT	Rollins 1993
Sample	**Species**	**Collector**	**Location**	**Nomenclature**

34	L. montanum Nutt. Ex Torrey and Gray var. montanum	Rollins 1723	N of Vernal, Uintah Co., UT	Rollins 1993
35	L. montanum var. montanum	Rollins 7983 (GH)	Montrose Co., CO	Rollins 1993
36	L. montanum var. montanum	Dorn 10450	Soda Springs, Caribou Co., ID	Rollins 1993
37	L. montanum var. montanum	Lichvar 8532	Twin Bridges, Elko Co., NV	Rollins 1993
38	L. montanum var. montanum	Lichvar 8516	Powderhorn, Gunnison Co., CO	Rollins 1993
39	L. montanum var. montanum	Lichvar 8531	Whirlwind Valley, Millard Co., UT	Rollins 1993
40	L. montanum var. montanum	Dorn 10471	Dillon, Beaverhead Co., MT	Rollins 1993
41	L. montanum var. montanum	Dorn 10493	Twin Bridges, Elko Co., NV	Rollins 1993
42	L. montanum var. montanum	Dorn 10471	Dillon, Beaverhead Co., MT	Rollins 1993
43	L. montanum var. nevadense Rollins	Holmgren 1322 (GH)	Pine Forest Range, Humbolt Co., NV	Rollins 1993
44	L. montanum var. stellae Welsh & Revel	Fertig 24136	Fredonia, Coconino Co., AZ	Rollins 1993
45	L. montanum var. tenellum (L.O. Williams) C.L. Hitchc.	Lyons 10, 621	Pine Creek, Gunnison Co., CO	Rollins 1993
Sample	**Species**	**Collector**	**Location**	**Nomenclature**

46	L. montanum var. wyomingense	Rollins 2262 (GH)	Green River, Sweetwater Co., WY	Rollins 1993
47	L. montanum var. wyomingense (C.L. Hitch.) C.L. Hitchc.	Peteron 83-231 (GH)	Moffat Co., CO	Rollins 1993
48	L. montanum var. wyomingense	Lichvar 8538	Lonetree, Uinta Co., WY	Rollins 1993
49	L. montanum var. wyomingense	Dorn 10500	Lonetree, Uinta Co., WY	Rollins 1993
50	L. nanum S. Wats.	Lichvar 8533	Jake's Valley, White Pine Co., NV	FNA*
51	L. nitidum Nutt. Ex Torry and Gray	Rollins 7801 (GH)	Riverside Co., CA	FNA
52	L. ostleri Welsh and Goodrich	Ostler 1415	Beaver Co., UT	FNA
53	L. ostleri	Fertig 24080	Grampian Hill, Beaver Co., UT	FNA
54	L. papilliferum (L. Henderson) A. Nels. & J.F. Macbride	Debolt 1010 (GH)	Kuna, Ada Co., ID	FNA
55	L. papilliferum	Debolt 1145 (GH)	Kuna, Ada Co., ID	FNA
56	L. papilliferum	Lichvar 8526	Mountain Home, Ada Co., ID	FNA
57	L. papilliferum	Macbride 880	Emmett, Canyon Co., ID	FNA
Sample	**Species**	**Collector**	**Location**	**Nomenclature**

58	L. paysonii Rollins	Payson 2699 (GH)	Snider Basin, Sublette Co., WY	FNA
59	L. thurberi Wooton	Lichvar 8511	Lordsburg, Hidalgo Co., NM	FNA

*Flora of North America (FNA)

CHAPTER 3 FIGURES

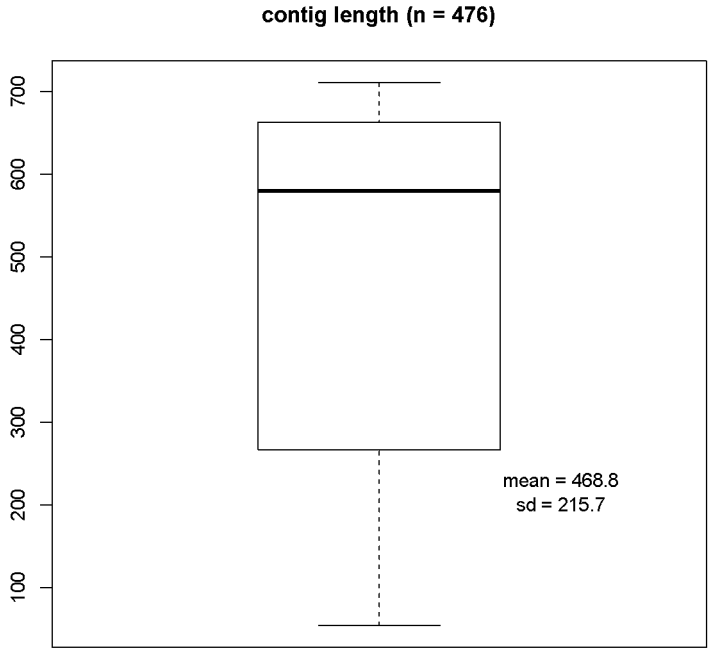

Figure 3.1. Bar plot showing the variation in assembled contig length (i.e., after assembling the two reads, forward and reverse for each sample).

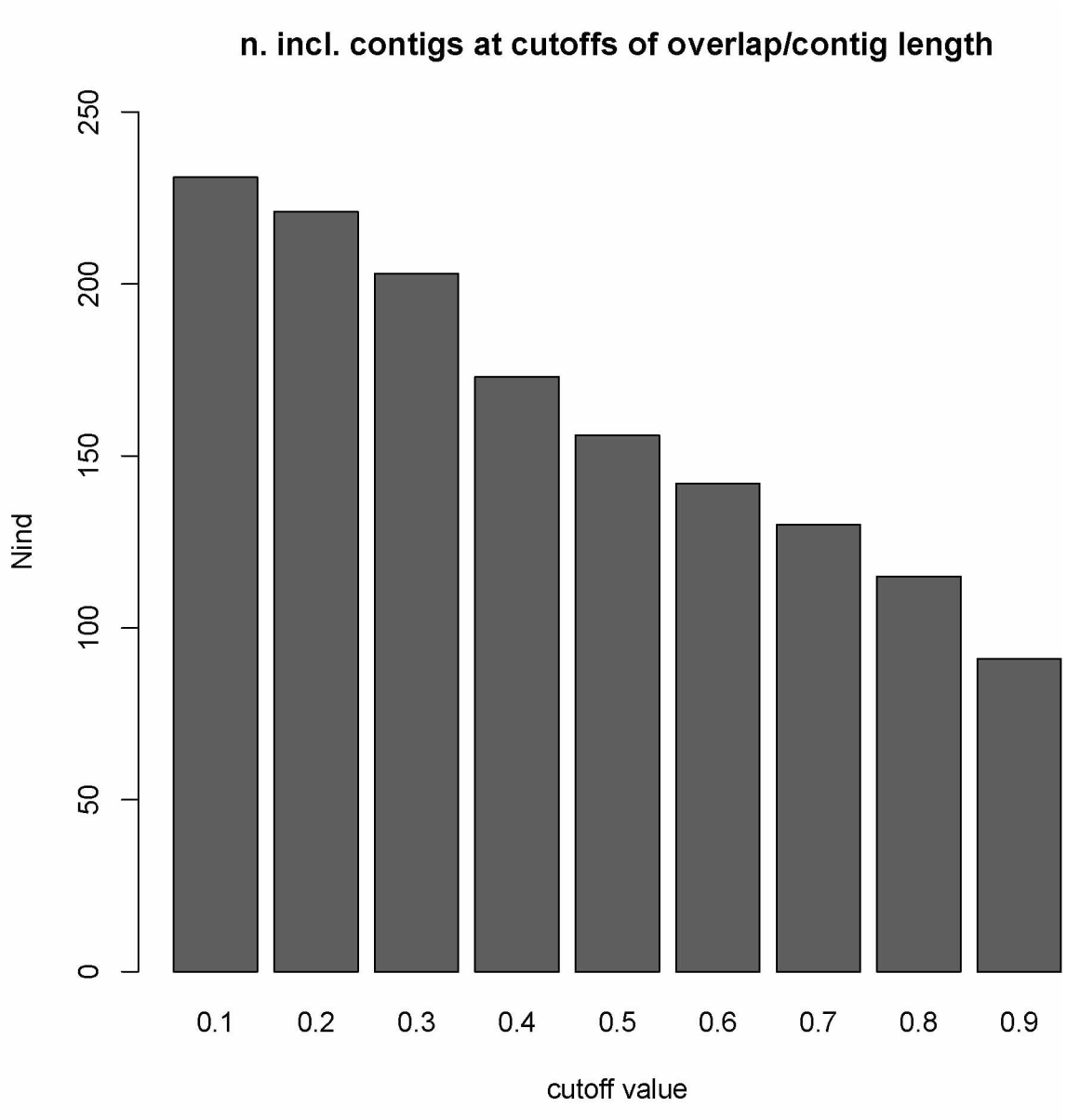

Figure 3.2. Incl. contigs at cutoff of overlap/contig length.

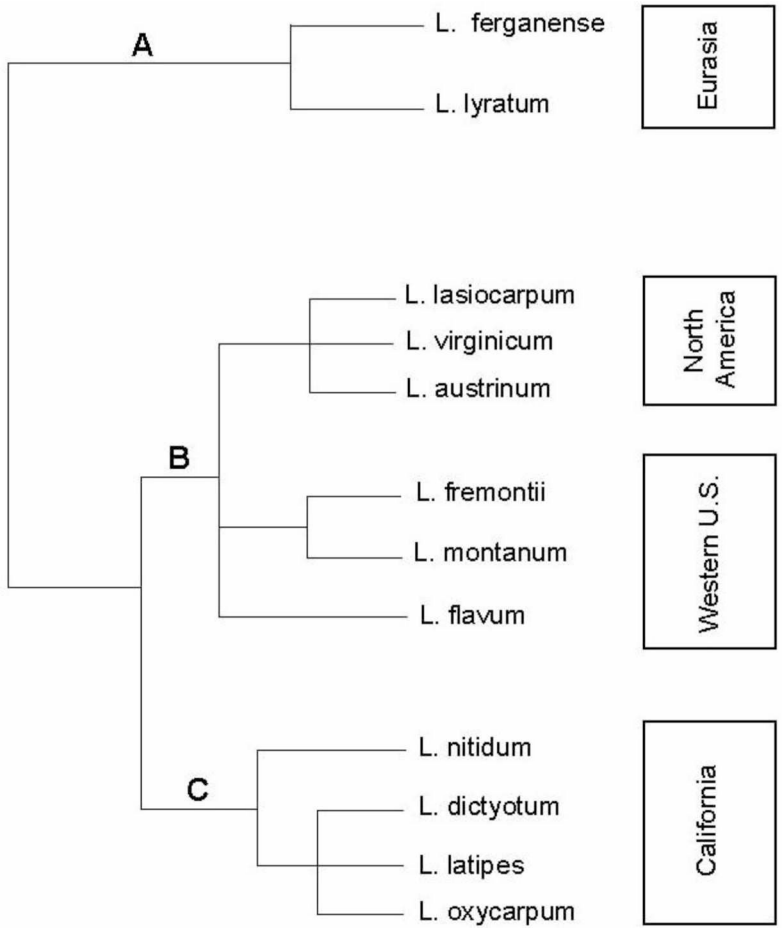

Figure 3.3. ITS analysis of western United States Lepidium (modified from Mummenhoff et al. 2001).

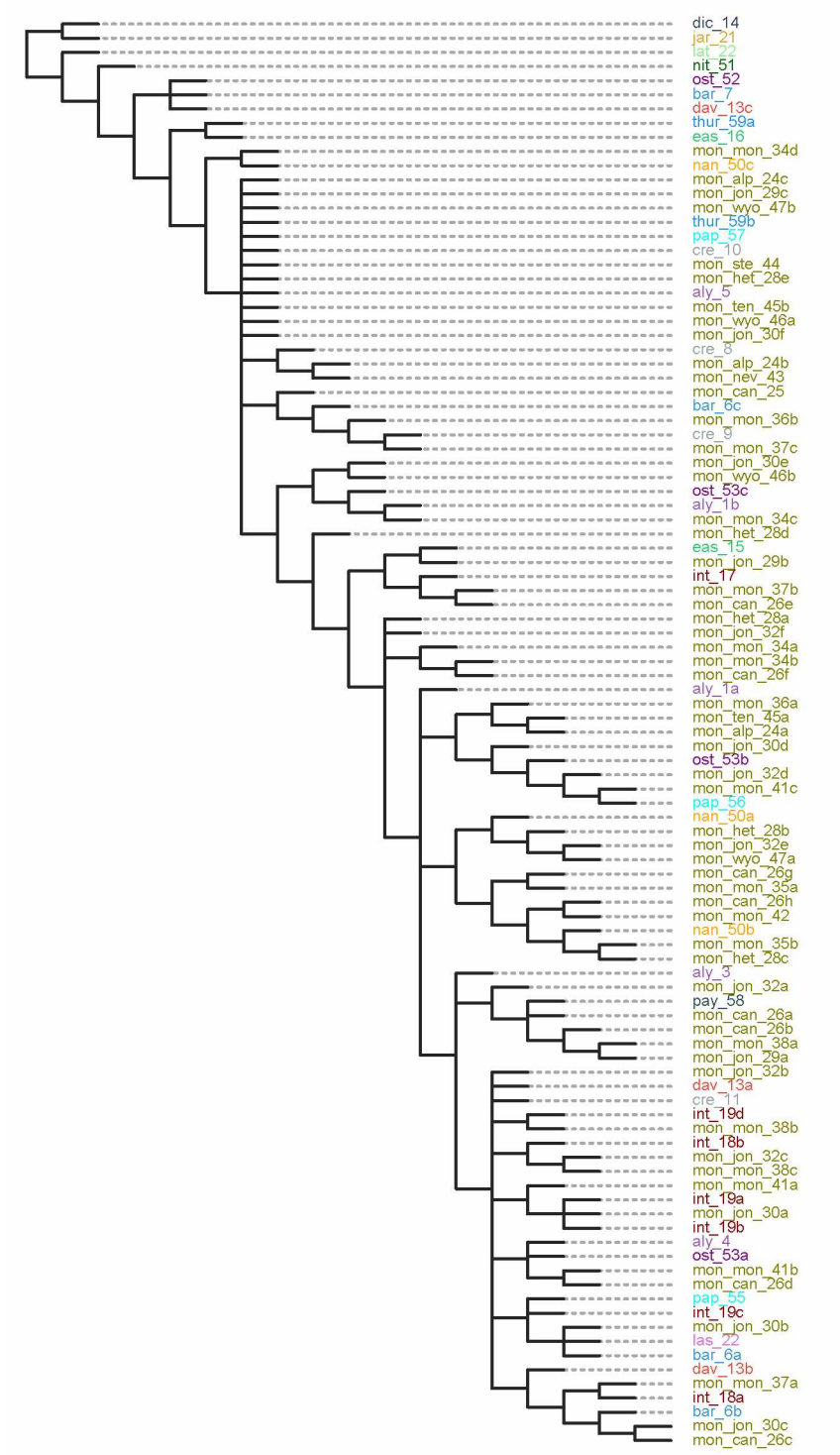

Fig 3.4. ITS tree

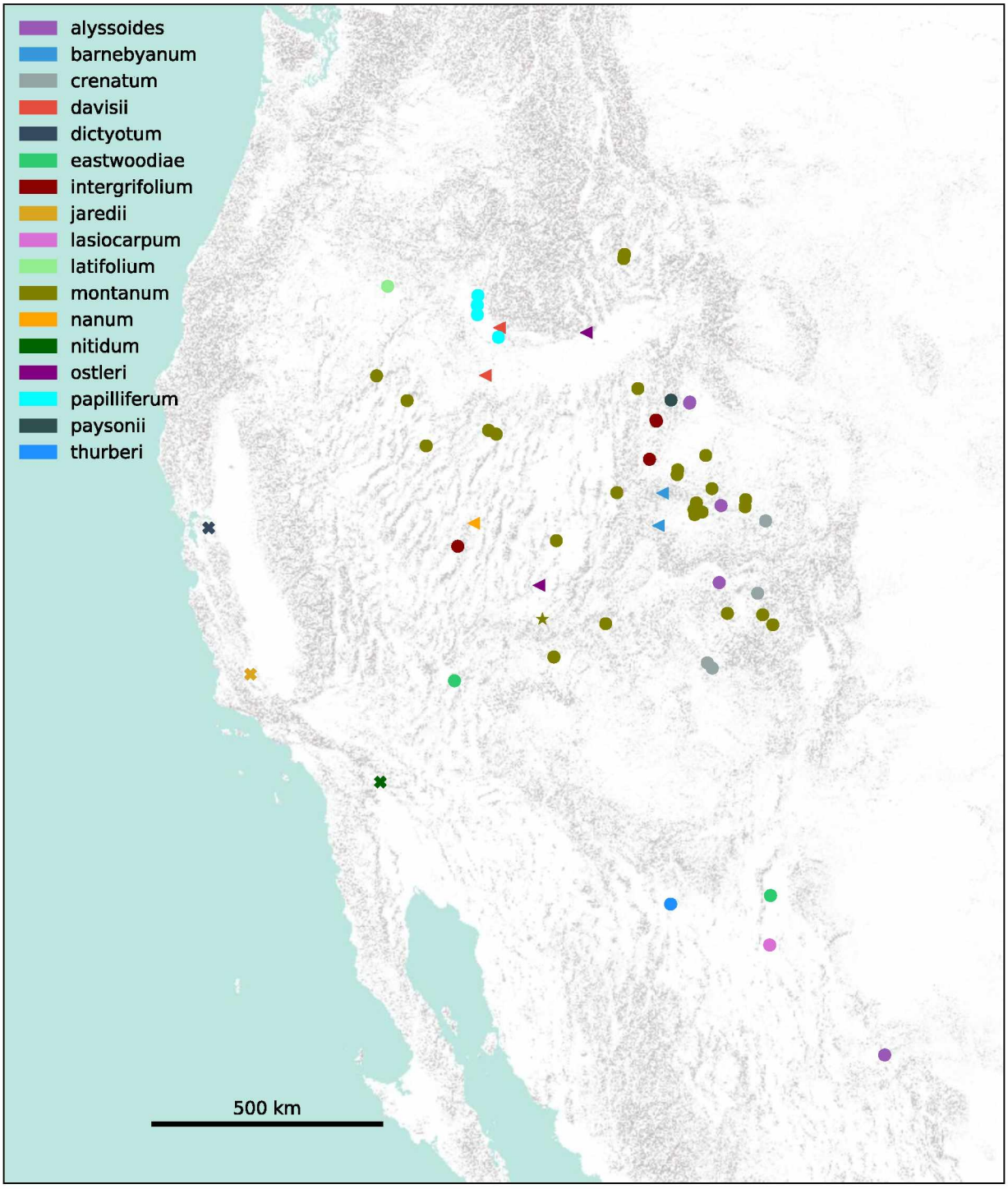

Fig 3.5. Map of western Lepidium

GENERAL RESULTS

Rapid and radiant speciation in the Brassicaceae evolution, as pointed out by Franzke et. al (2011), is supported by a strong phylogenetic congruence when based on nuclear rDNA ITS (Bailey et al. 2006), chloroplast ndhF (Beilstein, Al-Shehbaz, and Kellogg 2006) and nuclear phyA (Beilstein et al. 2008) sequence data in the basal part of the family. Alternatively, the lack of some basal resolution in phylogenetic trees could be the product of "among-tree conflict," possibly caused by processes such as recombination or ancient hybridization (Franzke et al. (2011). Within the family, genetic clarity between tribes, genera and species is a common issue. Rapid expansions of genera and species in the Brassicaceae is common within many species groups including Lepidium. To evaluate relationships and species concepts within the Intermountain Lepidium, a variety of both morphological and molecular data was used.

The most problematic section within Lepidium in the United States is the Intermountain group, particularly the L. montanum group. In Chapter 1, when using morphological traits, the Intermountain species sorted into reasonable morphologically-based clades of sister species using a discrete character parsimony algorithm (PARS) (Felstenstein, Univ. Washington; http://evolution.genetics.washington.edu/phylip/doc/pars.html). But in treating western U.S. species, both Rollins (1993) and Holmgren et al. (2005) remarked about the lack of understanding for the relationships that exist between L. barnebyanum and L. davisii, L. papilliferum and L. montanum, and the treatment of L. huberi through the L. alyssoides branch of species. However, all of these species cluster nicely when based on morphological features. Statements about relationships between select species made by Rollins (1993) and Holmgren et al. (2005) are reinforced when noting the incongruency between the cladogram and the preliminary nuclear ITS results in this study (Chapter 1). These clearly indicate that some of the species noted by these authors were more closely related to other species as identified using phylogenetic approaches.

In three phylogenetic trees using different genes, there are incongruences between several clades. The ITS phylogenetic relationships of seven western U.S. species sorted into two separate clades (Fig. 1.1). Clade C, labeled California, contains four species with distribution patterns mainly

from the west coast of the U.S. and into Nevada. Clade B was a mix of California and other western U.S. species that aligned with more widespread and weedy species found throughout North America. In Clade B, L. flavum Torr. splits at an equal node that sorts sister species L. fremontii S. Watson and L. montanum Nutt. The cpDNA positioned L. fremontii, L. montanum, and L. flavum together in the same final branch in clade A (Fig. 1.2). Similar to ITS, cpDNA placed these three western U.S. species closer to more widespread species from across North America. Eurasian species also linked phylogenetically to North American Lepidium (Mummenhoff et al. 2001) in Clade A, including both western U.S. and more widespread North American species. The California-restricted Lepidium aligned in their own sub-clade. In ITS studies, the Eurasian species were a separate clade (Fig. 1.1). While Pistillata analysis (PI) shows some similar alignments it does divides the western species into two clades. In the PI phylogenetic alignment, western species of L. fremontii, L. montanum, and L. flavum were located in both major Clades A and C (Fig. 1.3). Again, L. montanum and L. flavum aligned with more widespread Lepidium of Clade A from North America. Likewise, L. fremontii and L. montanum aligned with other widespread Lepidium in the other major Clades C and D. Interestingly, L. montanum occurred twice, once in Clade A with L. flavum and again in Clade C with L. fremontii. The Eurasian species L. lyratum L. aligned with the western U.S. and widespread Lepidium of Clades A and B.

Typically, species with faint and weak morphological features that are difficult to tell taxa apart many times are a result of reticulate evolution or hybridization (Linder and Rieseberg 2004). Since the Intermountain Lepidium have both weak traits for distinguishing species, hybridization was suspected and ploidy levels were surveyed (Chapter 2). With the high abundance and widespread occurrence of polyploids, especially tetraploids, in the Lepidium surveyed from the Intermountain West, ploidy levels do not provide much insight for explaining morphological differences or geographic patterns. However, there are a few noteworthy observations.

Ploidy levels in Lepidium as a whole show that they are not unusual for a single taxon to have more than one ploidy level. Examples of species with more than one ploidy level are L. latifolium (diploid and hexaploid), L. integrifolium (tetraploid, hexaploid, and octoploid), L. montanum var. canescens (diploid and tetraploid), L. montanum var. jonesii (diploid

[Warwick and Al-Shehbaz 2006] and tetraploid), and L. thurberi (diploid and tetraploid). Of the seven varieties surveyed in the L. montanum complex, only var. cinereum and var. canescens had more than one ploidy level. Variety cinereum is reported as both tetraploid and hexaploid, and var. canescens as both diploid and tetraploid. The hexaploid reported for var. cinereum is from a collection of var. stellae, submerged into the concept of var. cinereum (Al-Shehbaz and Gaskin 2010). However, it is important to note that the results of this survey may not support a significant inference about the lack of correlation between ploidy level and taxonomic, geographic, or habitat group because the number of non-tetraploid species was so small. Historically, the use of chromosome numbers for classification purposes in vascular plant taxonomy has long been limited to supporting or defining the concept of a species. The shape and number of chromosomes are not weighed differently than any other morphological feature for taxonomic purposes. The lack of use of ploidy level to support species concepts is no doubt linked to the fact that over 70% of angiosperm plants have ploidy level increases in their evolutionary histories (Meyers and Levin 2006). Because multiple ploidy levels are common in most angiosperm groups, it would appear that little distinction among taxa could be obtained from the variability. Stebbins (1971) proposed that a young polyploid complex would contain many diploids and a few tetraploids. As the complex ages, more species would become polyploids and higher ploidy levels would develop. As time progresses, the ancestor diploids would ultimately die out, resulting in greater difficulty in describing their evolutionary ancestry. Almost all species in this survey were of higher ploidy levels, 90% being tetraploids. In addition, the 11 native North American species of Lepidium with reported chromosome counts were 90% dominated by tetraploids (Warwick and Al-Shehbaz 2006). Using Stebbins's widely accepted concept (Meyers and Levin 2006), the Intermountain West Lepidium complex can be described as an evolutionarily young to moderately-aged species group with a reticulate evolutionary past. The indication of a reticulate evolutionary past has been demonstrated in Lepidium by incongruences in gene trees (Bowman et al. 1999, Mummenhoff et al. 2001, 2004). Bowman et al. (1999) and Mummenhoff et al. (2001, 2004) suggest that most species that are polyploids in Lepidium are allopolyploid. On the basis of previous cpDNA and ITS sequence data, allopolyploidization has been strongly favored to explain gene tree patterns (Mummenhoff et al. 2001, 2004). The influence of a reticulate evolutionary past in the Intermountain West region in this genus, coupled with both high occurrence of polyploidy across all species and highly mobile

seeds that are easily transported by avian species to allow for mixing of populations and species (Al-Shehbaz 1986b), provides for a diverse array of polyploid genotypes (Soltis and Soltis 1991). This evolutionary background may be setting a challenging stage for adequately classifying these native intermountain species. ITS molecular research (Chapter 3) on Intermountain West Lepidium led to DNA sequences that were analyzed and gene trees developed. Without gene trees, these flow cytombservations and indications of input of different parental DNA stock based on variable sizes of DNA content cannot be conclusively resolved. Likewise, with no association of geographic ploidy levels with geographic ranges or habitats and with variability of DNA content between Lepidium taxa, mere ploidy levels may not be useful for taxonomic purposes. However, ploidy levels did indicate the need for the construction of gene trees to adequately treat these diverse native species of Lepidium in the Intermountain West.

Results in Chapter 3 are difficult to interpret and the patterns manifest several known issues in using ITS as a single marker in closely related species. Feliner and Rosellio (2007) expressed concern that non-cloned ITS sequences retrieved from PCR product share many of the same target priming sites in one or more loci which can cause consensus ITS sequences. So, when non-cloned ITS sequences are used as raw data in reconstruction of phylogenetic relationships, it acts as a molecular phenotype from which the genotype can't always be inferred. Other drawbacks of ITS data include that there are hundreds or thousands of ITS copies spread across the genome Alvarez and Wendel (2003). Inferring phylogenies from ITS and multigene families can lead to incorrect results because there is variation within the different repeats in the genome Coleman (2003). Buckler et al, (1997) provided evidence that the variation among ITS sequences occurs mainly in hybrids and polyploids. These basic points about the variation within ITS sequences across the genome with concerted evolution make phylogenetic interpretations even more difficult (Poczai and Hyvönen 2010), in turn leading to different classes of ITS (Buckler et al. 1997, Wendel et al, 1995, Soltis and Soltis 1991, and Ritland et al. 1993). Differences from concerted evolution, hybridization and polyploidy, and repeats of ITS with different gene families, can cause intra-individual paralogues among sequences of an individual and can be maintained and shared with other species causing problems in phylogenetic analysis.

The ITS phylogenetic tree developed from this study (Fig. 3.3) amplifies many of the issues of using ITS for closely related species. A modified and reduced version of Mummenhoff et al. (2001) ITS global survey of the genus shows US west coast (California) as a different clade than the western group with its intermountain clade (Fig. 3.1) with the intermountain region L. montanum anchored with this separate clade.

As the outgroup in this study, species from the west coast of the United States in the California clade sorted separately as a clade and also as our outgroup in our ITS tree. Diploid taxa from Lichvar (2015) of L. ostleri, L. thurberi, and L. montanum var. canescens showed a weak association of tightly clustered clades nearer the root of the tree. But samples of these taxa also have scattered occurrences in other parts of the tree. In part, the ITS sequences of the diploids tended to show relationships with samples from the southern and western reaches of the study samples (Fig. 3.4).

Geographic patterns inferred from sequences for related taxa was faint for several groups (Fig. 3.5). For example, L. heterophyllum more typically located in the Oregon, Washington and Idaho region has an outlier population near Durango, CO with overlapping geographic ranges with L. montanum var. neeseae, L. montanum var. nevadensis, and L. crenatum and other L. montanum varieties. But these overlapping distribution patterns are weak and provide no inferences other than faint geographic overlapping ranges associated with most of the L. montanum varieties. Smith et al. (2009), using various gene markers, discussed eastern and western clades of patterns in taxa of Lepidium they surveyed. Their samples were limited to a smaller region of the Intermountain West than the broader range of samples surveyed here. Those patterns may be less meaningful when more of the intermountain taxa are included in an analysis.

Another pattern observed in this study is the proximity of caespitose entire leafed species generated in the ITS tree. The genus has the following narrow endemics with mostly linear entire basal leaves, as L. ostleri, L. barnebyanum, L. nanum and L. davisii. These species are not sympatric in distribution nor do they occur in similar habitats. L. davisii is found on clay vernal playas in parts of ID and NV. L. ostleri is found on limestone gravel opening in ponderosa pine

forest in Utah while L. barnebyanum is located on white shale ridges in Utah. Interestingly these three species, and also L. nanum, are generally all caespitose with entire leaves that tend to be slightly fleshy. This is a distinct growth form within the genus and in the ITS tree in our study. These taxa usually are in the same clade or even on closely related branch tips. Though inferences from the ITS analysis are weak, they indicate possible relationships between these species.

In the intermountain west, the species that is most commonly associated with all clades and branches is L. montanum. This species starts to make its appearance near the root of the tree and is scattered throughout all branches and clades. This taxon has a relationship with all other species and varieties in this region, which suggests introgression into many related taxa.

GENERAL CONCLUSIONS

Though the ITS molecular efforts did little to resolve either species relationships or a better insight to the nomenclature and taxonomy of Lepidium, several key points have been made. The use of morphological features by previous taxonomic authorities stood as a way to classify and distinguish Intermountain species of Lepidium. However, in limited ITS analysis using the same groups of species as in the morphological cladistics analysis, there were incongruences between the two approaches. The molecular analysis showed that the relationships were not similar in most cases as to the clustering of clades in the morphological approach. This comparison indicated possible frequent hybridization between many of the species. Yet, the ploidy level analysis showed no relationships within very difficult to classify taxa such as L. montanum varieties or any other morphological features or geographic patterns. Tetraploids were a common ploidy level with a few diploids and one hexaploid. But none of these ploidy levels were connected to any meaningful patterns to provide insights into hybridization causing faint and weakly distinct features useful for taxonomic purposes. And because of various issues with using one gene analysis and issues associated with ITS within the genome, no conclusive evidence was obtained to reliably observe patterns of divergence or relationships amongst Lepidium species. One possible species associated with parental stock involved the divergence of many species is L. montanum. In ITS analysis, this species is associated with each clade or pattern of association. It is found in basal groups as well as on multiple tips of many clade branches. Understanding this species using molecular methods is key to improving the classification of Lepidium in the Intermountain West.

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